AN INVESTIGATION INTO THE REGULATION OF SEGMENT NUMBER IN THE LEECH

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I declare that this thesis was composed by myself. Contributions of others to the work are clearly indicated.

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"...on peut regarder une pièce d'un puzzle pendant trois jours et croire tout savoir de sa configuration et de sa couleur sans avoir le moins du monde avancé: seule compte la possibilité de relier cette pièce à d'autres pieces, et en ce sens il y a quelquechose de commun entre l'art du puzzle et l'art du go..."

(George Perec,
La vie mode d'emploi)

Abstract

The control of the number of segments is a problem posed in all segmented phyla: how can there be generation of a constant number of body parts? Here I have studied the leech embryo as an example of an animal where the number of segments is regulated. The leech develops in a stereotypical manner and gives rise, during the cleavage stage, to 5 pairs of teloblasts. These cells divide in a stem-cell manner, producing blast cells, the founders of the segmental body plan. The blast cells are produced one after the other, and stay ordered in such a way that the most anterior blast cells are the oldest and the most posterior the youngest. As they move anteriorly, the bandlets or columns of blast cells from the different lineages converge to form the germinal band. This results in an anteroposterior gradient of development. In most teloblast lineages, one blast cell populates one segment equivalent. However, more blast cells are born than there are segments.

I have been using lineage tracing to study the fate of the cells that are eliminated in the process of segment regulation in the leech. In combination, I have been looking for genes that could be involved in such a process of number counting and boundary formation.

My results confirm the presence of two types of blast cells, the segmental cells (that go on to form the segmental body) and the supernumerary cells (that die). They suggest (against what was previously thought), that blast cells do not need to be in contact with neighbours of the other lineages (i.e. the germinal band) to divide, but their presence in the germinal band is required for making segments. My results also show that the pair-rule gene *hairy* might not be implicated in setting up the boundary between segmental and supernumerary cells, but that the pair-rule gene *patched* might be

indirectly implicated by being part of a signalling pathway between the segmented tissue and the overlaying provisional epithelium.

I propose a model whereby the fate to become segmental is partially determined at birth and partially determined by the environment. The cells are able to move on to the next step of their cell-autonomous program only if they receive the correct environmental signal.

Chapter I: The leech as a model for sequential segmentation

Introduction

Since the evolutionary step from single cell to multicell organism (protozoa to metazoa transition), the different cells from a single organism have had to become organised. The processes of differentiation, spatial patterning and change of form, are essential for development into an organised structure (Wolpert, 1990). Before form can arise (by morphogenesis), there must be spatial arrangement of cells with different cellular differentiation. The problem of spatial arrangement, or pattern formation, is concerned with how differentiation occurs at a specific time at a specific place. Cells start by being different from each other in their cell state (Slack, 1991), defined by the expression of a specific combination of molecules.

Among the infinite variety of patterns that could be formed during development, very few are actually encountered. On the other hand, some patterns, such as segmentation, can be found time and time again in different organisms, and in the development of different structures and tissues. It is important to realise that the variety of patterns that we can observed today in different organisms, are the result of selection and constraints exerted on the adults and on the embryos.

This thesis is concerned with the formation of the segmental pattern and the control of the number of segments. In this introductory chapter, I will be describing segmentation as a pattern, and comparing segmentation in different organisms. Finally, I will argue for the validity of such a comparison, and whether the leech can be used as a model organism.

Defining segmentation

The importance of segmentation in the building up of the body plan cannot be overestimated. Segmentation is found in three different phyla, the arthropods, the vertebrates and the annelids, all of which show a morphological periodicity along their body axis. One definition of segments, provided by Stent (1985) is that they are "a module of serially iterated structures [...] differing to various extent from the basic ground plan".

Of the 3 segmented phyla, the first one is hailed as the success of our era (the arthropods; in 1995 alone, 1395 entries on BIDS) because it has the most species on earth, and has adapted to an amazingly large variety of ecological niches; the second one interests us particularly because we are one of them (the vertebrates; in 1995 alone 91816 entries on BIDS) and because the large brains of species of this phylum have been hailed as another evolutionary success. Finally, the 3rd phylum is hailed as a success by the fishermen who would be at a loss for bait if the earthworm didn't exist (annelid; in 1995, only 62 entries on BIDS)!

It is worth noting that segmentation is not just an interesting and intriguing pattern for the observer. Within the segmented phyla, it happens that the phylotypic stage, i.e. the stage of development at which the members of the phylum show the maximum similarity (Sander, 1983), is the stage of segmental completion (Slack, et al., 1993). The fact that the stages leading to segmentation can be quite different suggests that segmentation can be achieved by a variety of mechanisms. Since it is so easily achieved, it is possible that the segmental pattern confers a low energy state, and is the result of strong developmental constraints.

The most widely accepted phylogenetic relationship between these three phyla places the arthropods on a common branch with the annelids, unrelated to the vertebrates (Anderson, 1973; Lake, 1990). There is relative

agreement that arthropods and annelids have evolved from a common segmented ancestor, but that segmentation in the vertebrates is not homologous (Clarke, 1964). Indeed, it is difficult to find a definition of segmentation that would encompass the particularities of each species.

For a characteristic to arise more than once, it has to be achievable through few steps (mutations) to increase the chances of appearing, and it has to be advantageous or neutral in order to be selected for (fixation). It can be argued that advantage is conferred to segmented organisms because segmentation divides the body plan into smaller morphogenetic fields, all identical at first, therefore the total information required for forming the whole body plan is reduced. Also, once the body plan is subdivided, each subunit is more or less independent of the other. Patterning of the whole body plan can take place independently in the different subunit, restricting any error during further development to a subset of subunits rather than spreading it to the whole body plan.

Control of segment number

A large number of segmented animals possess a determined number of segments, specific to their species, familly, genera or group. For example, chick have between 50 and 53 somites (Sanders, et al., 1986); all mammals have 7 cervical and 29 precaudal vertebrae; *Drosophila* has exactly 3 thoracic and 8 abdominal segments; leeches (Hirudines) have exactly 32 segments. These figures are much more consistent than the variation of the body size (Maynard Smith, 1960), suggesting that there is a control of segment number relative to the size: segments vary in size rather than in number to fit a highly variable body size. Furthermore, unless developing in extreme conditions, or as a result of manipulation, these animals regulate their number of segments such that any variation happening in a normal environment can be interpreted as genetic.

What accounts for such regularity? It is possible that developmental constraints fix the number of segments in each species. That is, given the properties of the developmental system, there are only a limited number of segments their body would ever support. In this case, the number of segments would be a secondary consequence of other events in development. However, the evidence that the number could be modified in some experimental circumstances (see below), suggests that the constraints (if they exist) are not a limiting factor. Alternatively, the maintenance of uniformity might be due to natural selection, in which case there has to be a developmental mechanism that controls the number of segments. To distinguish between the two hypothesis (developmental constraints and natural selection) is not an easy task (Maynard Smith, et al., 1985), and in this thesis, I will only be concentrating on trying to find out if a developmental mechanism is involved in the control of segment number.

Why does segmentation need to be controlled? The most obvious reason would be that once segmentation arose, specialisation of the segments started occurring: subdivision of the vertebrae into different subsets of the vertebrate body; specialisation of the thoracic segments of Drosophila; and less conspicuously, reproductive segments in the annelids. For diversification of the segments to occur accurately, each segment had to be present.

How might segment number be controlled? It is convenient, to start with, to suppose that the control arises from a simple set of rules. Theoreticians have tackled the question from two different angles, posing the problem as either a "French Flag problem" (Wolpert, 1968) or a "Counting problem" (Maynard Smith, 1968). Every other simple set of rules would relate to either one or the other.

In the first approach, Wolpert wonders how an animal that varies in size can not vary in its number of subdivision. His theoretical solution is

that the organism would measure the field to be divided. It is easy to imagine a system whereby the measuring of the field would be concommitent with the allocating of "positional information"(Wolpert, 1969). Wolpert suggests as a possible solution a chemical gradient which could both measure and allocate positional information: a specific positional information is given to the part of the field situated between two specific concentrations (thresholds) of the chemical (Figure I.1A).

Maynard Smith proposes a rather different approach. His solution uses a counter, a method whereby the addition of tissue in blocs (segments) is recorded, fed back into the system until the required appropriate number of segments is reached (see Figure I.1B). In this system, regulation of the number of segments in a variable size organism can be achieved if the size of the segments is proportional to the size of the future body. This requires that the potential body size be known before the animal starts segmenting.

To the control of segment number problem, Wolpert and Maynard Smith suggest two completely different solutions, requiring different conditions from the start. Are these conditions met by segmental systems?

The stages leading to complete segmentation can be dramatically different even in closely related species. Can these different species still use the same mechanism to regulate their number of segments? In the following paragraphs, I will analyse segmentation in different systems to find out how well they fit either of the two models. I shall start by examining the leech and other annelids because this is the system used in this thesis. I shall then examine other species, specifically the long germ band Drosophila and other insects, and several vertebrates. Particular attention will be paid to similarities found across phyla.

Comparing the different segmental systems

Segmentation in animals can be classified as occuring either "by subdivision" or "sequentially" (Stern, 1990), and these two types reflect

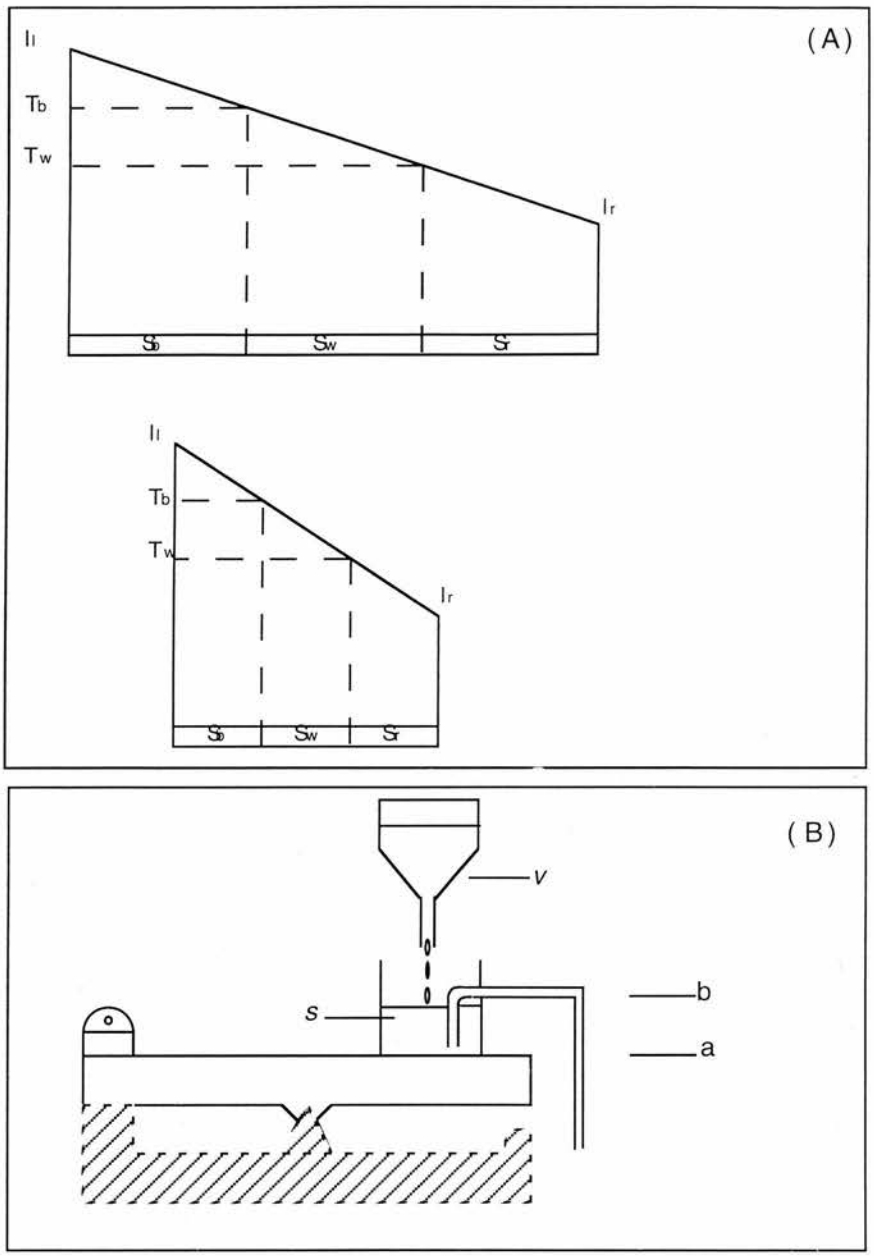


Figure I.1 Two methods by which embryos could regulate their number of segments (A) Wolpert's French Flag method. The pattern is formed by means of a gradient established between the right and the left limits of the field (ll and lr), with fixed concentrations at each end. As long as the concentration stays linear between these two end-points, the proportion of Sb, Sw and Sr are conserved in a smaller field. In this model, a smooth gradient is converted into 3 discrete domains, with the help of 2 threshold levels. The model could be extended to more fields (segments) either by increasing the number of thresholds, or alternatively by subdividing each field again. (B) Maynard Smith's Ratio Counter. In this system, the volume of liquide required to fill the upper vessel *V* is n times that required to fill the lower tank *S* from a to b. When the lower vessel is almost full, it tips the the seesaw. This machine breaks up a continuous process into a number of discreet events (segments). However, unless *S* varies proportionally when *V* varies, the machine does not regulate for size variation.

segmentation according to the French Flag and the Counting Machine respectively.

Segmentation by subdivision consists of establishing limits within an existing tissue, all more or less simultaneously. The tissue is polarised antero-posteriorly by a chemical difference, but the polarity does not continue to develop once segmentation begins. The main condition, then, for this type of segmentation, is that the length of the segmental field can be estimated before segmentation begins. In this case, the final length of the segmental tissue dictates the size of the segments.

In the sequential mode of segmentation, the tissue develops following an antero-posterior time gradient of development, and the segments appear one after the other, as the tissue matures. All the cells may not be born at the time segmentation starts. In this case, it is the number of segments and their size that dictates the final length of the segmental tissue.

Development and segmentation in the leech

The Annelids are the segmented phylum par excellence. Annelids comprise the Polychaetes and the Oligochates classes, with the Hirudinea class or subclass being closely related to the Oligochaetes. All the Annelids are segmented, and they differ from the other segmented phyla (Arthropods and Vertebrates) by the fact that the segments are still often recognisable in the adult and that in some families, the segments are identical. Leeches (Hirudines class) are among the annelids where segmentation is overt, and where the segments generally show little morphological variation. The leech can be considered representative of the way annelids develop, in that it follows a modified spiral cleavage that resembles that of other annelids (Sandig and Dohle, 1988).

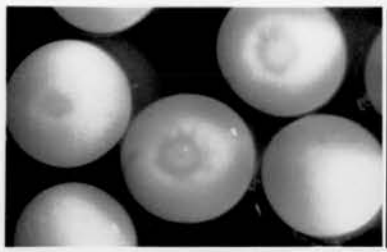
The leech has been an organism of choice for many years in the study of development. Its ubiquity, ease of manipulation due to large cells, and small number of cells during early development, have led to there being

many descriptions at the cellular level, going back more than a century, when Whitman used *Clepsine* (now known as *Glossiphonia complinata)* as a model organism to study cell lineage in development (Whitman, 1878; Whitman, 1887; Whitman, 1892), and Retzius used *Hirudo medicinalis* to study developmental neurobiology (Retzius, 1891). The large cells and the deterministic cell lineage meant that it was possible to recognise the cells of the fate map from the early stages of development.

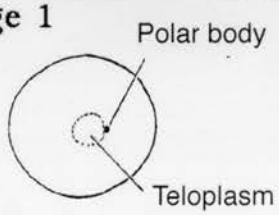
More recently, the leech has received renewed attention for different reasons. Lineage tracers have been used to follow cells in more detail, allowing more precise fate maps to be drawn (Weisblat, et al., 1978; Weisblat, et al., 1980). The leech, being an annelid, has also been used for evolutionary studies of segmentation, especially at the molecular level (Aisemberg, et al., 1993; Lans, et al., 1993; Nardelli-Haefliger and Shankland, 1992; Nardelli-Haefliger and Shankland, 1993; Patel, et al., 1989; Wedeen, et al., 1990a). The highly stereotypical lineage of the leech has prompted much research in the domain of cell determination (Blair, 1982; Blair, et al., 1990; Ho and Weisblat, 1987; Kostriken and Weisblat, 1992; Torrence, et al., 1989; Zackson, 1984). The leech ressembles another animal with very few cells, a highly stereotypic mode of development, and little regulation, namely, *C. elegans*. *C.elegans* is an unsegmented nematode worm, where signalling has been shown to be required in the determination of cell fate in many instances [in *C. elegans:* vulval determination (Han, 1992; Waring and Kenyon, 1991)]. Finally, the leech has also provided some ideas for the question of segment number regulation (Shankland, 1984).

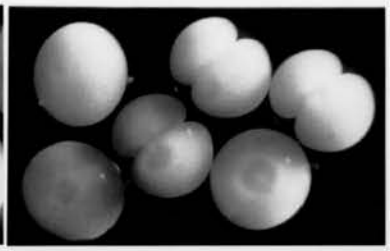
The development of glossiphoniid leeches is described in Figure I.2 and Table I.1. The oocyte is fertilized internally and released from meiosis upon egg deposition. The leech develops through a series of highly stereotypic cell divisions, allowing specific cells to be recognised from one embryo to the next by virtue of their relative position, size, shape, and

Figure I.2 The Development of Glossiphoniid leeches. **(A)** Photographs are of representative stages of development of *Theromyzon tessulatum*. The diagrams below each photograph are of the same stage, always viewed from the future dorsal side except the late stage 8. Refer to Table I.1 and text for a description of the stages. **(B)** More development of Glossiphoniid leeches. Confocal microscopy representations of the teloblast division stages. Embryos were labelled with a fluorescent nuclear marker. (a) stage 6a, dorsal view. The right M teloblast can be observed at the bottom of the picture(*M*). 3 ectoteloblasts (*t)* are obvious on the surface, and an M bandlet (*b*)can be observed, very close to the surface at the early stages of teloblast division. (b) dorsal view of late stage 7. The teloblasts (*t*) are burried more deeply, below the germinal bands. 4 bandlets can be observed in the germinal bands. The more intense blue dots (in the centre) represent nuclei of the micromeres on the very surface of the embryo. (c) lateral view of stage 7. The embryo is now viewed from the angle where the arrow is pointing in (b), and the reconstitution is not complete so that it is now possible to observe the teloblasts bu ried below the bandlets. (d) ventral view of late stage 8. The germinal bands have finished coalescing into the germinal plate. Segmentation starts being visible, see for example the grouping of the nuclei.

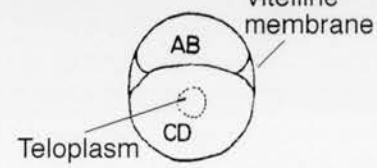


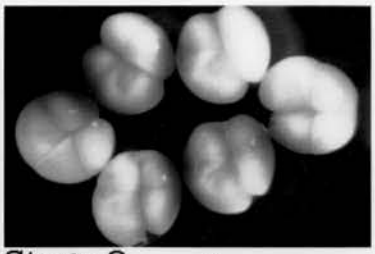
Stage 1



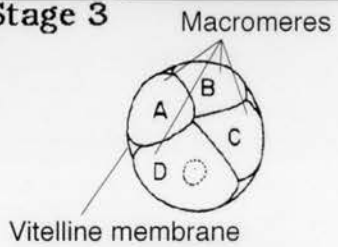


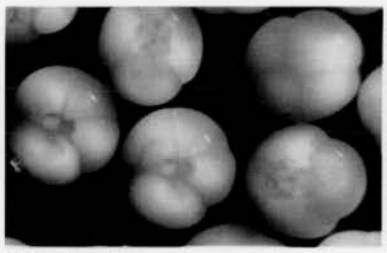
Stage 2



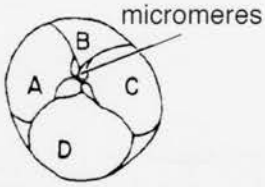


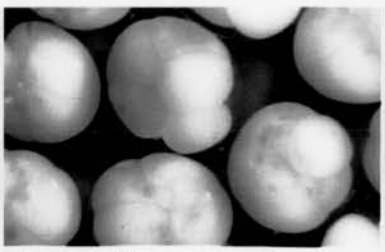
Stage 3



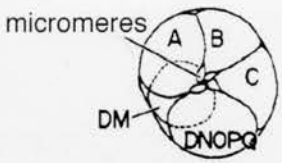


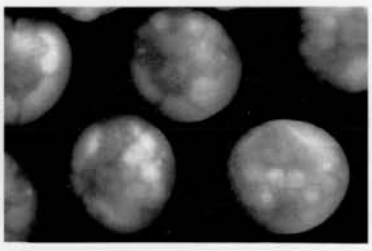
Stage 4a



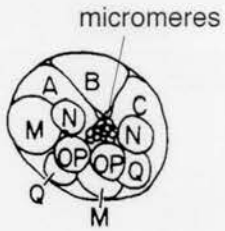


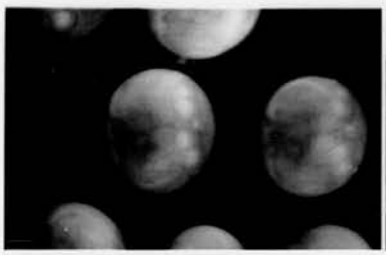
Stage 4b



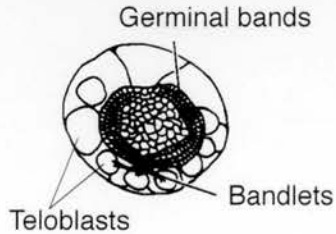


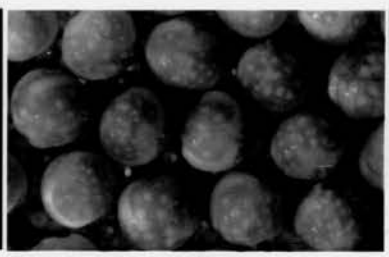
Stage 6b



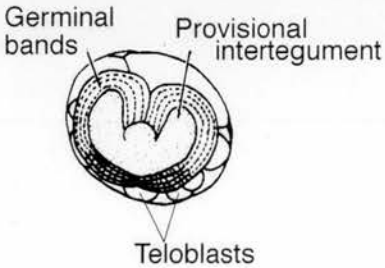


Stage 7 middle

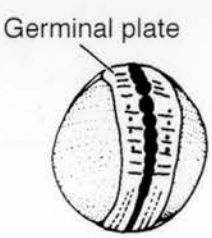




Stage 8 early



Stage 8 late



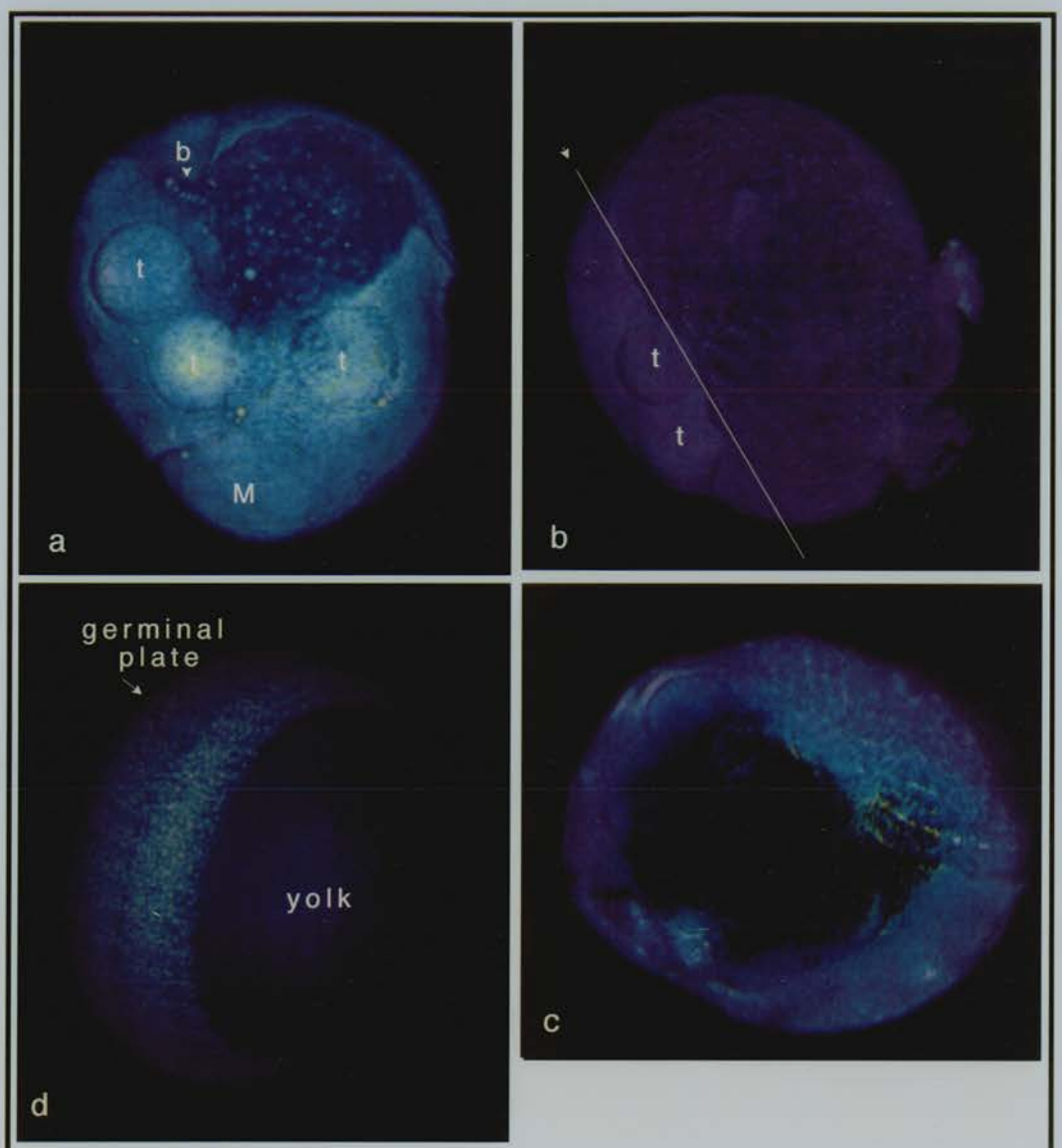


Figure I.2B

Stage	Description	Timing *T.r.* /*H.t.*
1	Uncleaved egg	hr 0 to 12 /hr 0 to 4.5
2	First cleavage. Formation of cells AB and CD	hr 12 to 15 /hr 4.5 to 6.5
3	Formation of cells A and B, and C and D	hr 15 to 25 /hr 6.5 to 8
4	Formation of the M teloblast pair:	hr 25 to 40 /hr 8 to 14
4a	Formation of the first 4 micromers a', b', c', d'	
4b	Formation of DM and DNOPQ	
4c	Formation of the M teloblast pair	
5	Formation of the NOPQ proteloblast pair	hr 40 to 45 /hr 14 to 17
6	Formation of the N, O, P and Q teloblast pairs:	hr 40 to 90 /hr 17 to 30
6a	Formation of N and OPQ	
6b	Formation of OP, and Q	
6c	Formation of O/P and P/O	
7	Formation of the germinal bands	hr 90 to 160 /hr 30 to 78
8	Coalescence of the germinal bands	hr 160 to 230 /hr 78 - 122
9	Completion of formation of the nerve cord ganglia	hr 200 to 320
10	Segmentation of the gut	hr 350 to 850

Table I.1: Developmental stages of Glossiphoniid leech embryos, with timing in *Theromyzon rude* (*T.r.*) at 14°C (Fernández, 1980) and *Helobdella triserialis* (*H.t.*)at 25°C (Bissen & Weisblat, 1989; Weisblat, et al., 1980). The timing of *Hellobdella robusta*, the leech dominantly used in this thesis, is very close to that of *H. triserialis*.

colour (cytoplasmic content). During the early cleavages (until stage 6), three different types of identifiable cells are produced: the macromeres, the micromeres and the teloblasts.

The macromeres (A, B, and C become A''', B''', C''' upon dividing to give 3 micromeres each), do not divide further in early development, although there is some evidence that later in development they undergo many nuclear divisions upon forming the gut (Nardelli-Haefliger & Shankland, 1993; Weisblat, et al., 1984). They provide support during the gastrulation movement of the bandlets (see below) and the micromeres. They are usually considered to be inert cells, but their role in the early stages of development has not been studied enough to know whether this is true (see stages 3, 4a, 4b and 6, Fig.I.2A).

The micromeres are small cells originating from the macromeres or the teloblasts and their precursors. Micromeres form a group of cells at the dorsal pole of the embryo (see stage 4a, 4b in Fig. I.2A). They divide actively, covering the dorsal part of the embryos including the bandlets (see stages 6b, 7 middle Fig. I.2A) and undergo a movement of gastrulation during stage 8 (see Fig.I.2A), over the macromeres (Ho & Weisblat, 1987; Smith and Weisblat, 1994). These cells contribute to non-segmental tissue such as the provisional epithelium of the body wall of the leech, the epidermis of the prostomium, and neurons of the supraesophageal ganglion (Ho & Weisblat, 1987; Weisblat et al., 1984).

The teloblasts all originate from the D cell, born at the second cleavage (stage 3, see Fig.I.2A). The D cell is special in that it contains most of the teloplasm, yolk-deficient cytoplasm segregated from the yolk before the first cleavage (Fernández, 1980; Whitman, 1878). The teloplasm is thought to contain the information responsible for the formation of the ectodermal and mesodermal lineages (Astrow, et al., 1987) and consists of a large number of organelles, particularly mitochondria. The D cell cleaves

several times to give micromeres and 5 pairs of bilaterally symmetrical stem cells, the teloblasts (stages 4 to 6, see Table I.1 and Fig. I.2). There is one mesodermal teloblasts (M), and 4 ectodermal teloblasts (N, O/P, P/O and Q). These divide repeatedly and regularly, producing a line of blast cells, or bandlet (stages 7 to 8, Fig. I.2A). These blast cells are the founder cells of the segments. They divide in a highly stereotypic manner, with all the blast cells of one lineage giving rise to similar clones from one segment to the next. Each blast cell clone (or 2 blast cell clone in some lineages) populates one hemisegmental complement. This means that although all the blast cells from the same teloblast produce similar clones of descendents, these are not constrained to one segment (Figure I.3). This has the consequence that in order to generate exactly 32 segments, each teloblast has to produce 32 founder cells (or 64 in the lineages with 2 blast cell clone per hemisegment).

The O, P and M teloblasts give rise to only one type of blast cells i.e. one lineage, but the N and Q teloblasts give rise to two types of blast cells. This means that each of the M , O and P teloblasts will produce 32 blast cells, and the N and Q will produce 64 to populate all the segments. The blast cells are arranged in their order of birth, in an antero-posterior gradient of development along the body axis and the bandlets, with the first ones already forming segments while the most posterior are still being born.

As the blast cells advance more anteriorly, the bandlets meet on each side, forming the germinal band. The germinal bands meet anteriorly, and as they progress in an enveloping movement around the embryo, they fuse antero-posteriorly, forming the germinal plate. In effect the germinal bands "zip-up" antero-posteriorly to form the germinal plate. The segmentation of the mesoderm becomes conspicuous first as the body wall muscles appear. At the end of stage 8, the germinal bands have coalesced entirely, and the nerve cord ganglia start being formed; at the same time the germinal plate

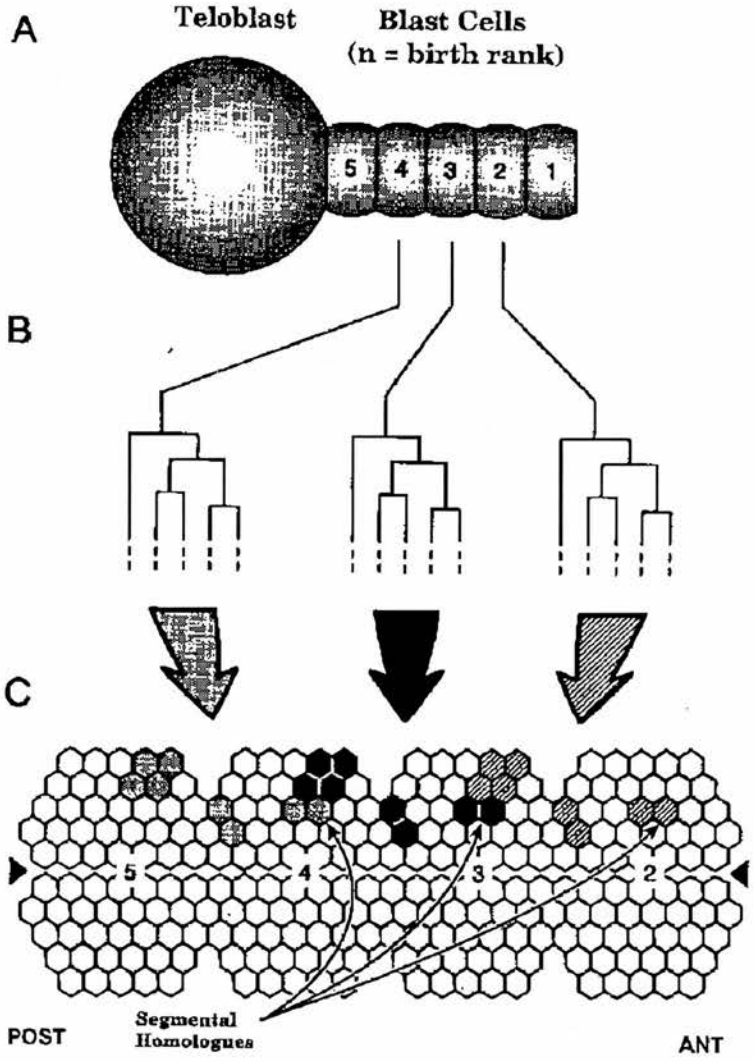


Figure I.3 Diagram depicting the way blast cell descendents span more than one segment. (A) Each teloblast divides repeatedly to produce a linear array of blast cell daughters which retain the order of their birth. (B) blast cell daughters of the same teloblast undergo very similar descendent lineages, and thus give rise to comparable clones of descendents situated in different body segments. (C) During normal development there is an invariant relationship between the blast cell's birth rank and the segmental location of its descendent clone. Note that the individual blast cell clones are roughly one segment in width, but distribute over two consecutive body segments and overlap at their edges. Individual segments are labelled with numbers, and arrowheads mark the midline (From Shankland et al, 1991).

extends laterally, making the body wall, and replacing the provisional epithelium provided by the micromere descendents.

Regulation of segment number in the leech depends in part on the correct number of blast cells being formed. Even in species such as *H. medicinalis* which has much larger macromeres and smaller teloblasts, and hence different morphogenetic movements, exactly 32 segments are generated through blast cells born from the teloblasts. However, in both species, the teloblasts do not stop dividing when the number of blast cells (i.e. segment founder cells) has reached 32. On the contrary, they continue dividing, producing more blast cells. These blast cells, not being required for making the correct number of segments, degenerate (Fernández and Stent, 1982; Zackson, 1982). So, although the leech appears in first instance to generate segments sequentially, suggesting that regulation of segment number is done by a counting machine, this is clearly not the whole mechanism. Regulation in the leech must be achieved both by a primary "rough" counting (the control of cell division of the teloblasts), and a secondary, more accurate counting of the segments which involves trimming off any extras. This trimming can either involve directly counting the segments or more generally regional specification (see below).

A constant and small number of segments is highly characteristic of the metamerism of leeches. This feature has been linked to the presence of a posterior sucker, which prevents the formation of a posterior growth zone as seen in the other Oligochaetes (Livanov, 1940). Another major characteristic of leeches is their high degree of heteronomy (diversification of the segments): cephalisation results from fusion of a number of segments, the clitellium results from modification of some segments, the animals have anterior and posterior suckers, the width of the body varies greatly in its separate parts (constricting towards the front end), and the segments are integrated into annuli althought the number of segments per annulus varies

along the body axis. Oligochaetes only share some of the heteronomy of the leech, namely cephalisation and formation of the clitellium. It is important to realise that leeches are therefore a highly specialised group, and might not be representative of other annelids or even Oligochaetes in every instance (Beklemishev, 1969).

Segmentation in related annelids

Eisina foetida, an Oligochaete with a relatively variable number of segments

Development in *E. foetida* is very similar to that of the leech, and segments are generated in the same way, by teloblasts generating segment founders (Storey, 1989a). The blast cells themselves follow a very similar pattern of divisions. However, the final number of segments in the earthworm is highly variable, with adults having between about 67 and 115 segments, with an average of approximately 100 (Moment, 1946) . This variability is even larger when considering different populations, which suggests that the final number of segments in the worm depends strongly both on its genetic background and on environmental conditions.

The final number of segments in this organism may rely exclusively on the control of cell division of the teloblasts, although it should be borne in mind that there has been no extensive studies to ascertain whether any cell are eliminated, as in the leech. It is probable that this organism only possesses the "rough" primary control of cell division. That way, the final number of segments is directly correlated to the number of cell divisions the teloblasts have undergone. Although this number is much less regular than in the leech, some coordination is probably required between the teloblasts: they all need to stop dividing at the same time, because If one teloblast goes on dividing longer than others, it will produce blast cells that are unable to make segments. So, either all the teloblasts stop dividing after the same

number of blast cells, or the extra blast cells are eliminated. Either way, a secondary mechanism of regulation is require. In its control of segment number, the earthworm does not follow only a simple set of rules as described above.

The earthworm is also different from the leech in that it can regenerate ablated segments. It is likely, however, that the mechanism of posterior regeneration is different from that of generation of the original segments: when the posterior segments are ablated, the teloblasts are always ablated too. Studies where the teloblasts were ablated in the embryo have shown that the segmental tissue is generated by different ectodermal cells, and the teloblasts are not replaced (Storey, 1989b). The worms which have regenerated segments after ablation in the adult have a much more consistent number of segments (around 100), and this number does not depend on the number of segments of the individual *before ablation.* This suggests that after ablation, there is counting of the remaining segments in order to estimate how many now need to be generated. The segments generated are always smaller than the original, embryonic, segments, and it is interesting to note too that the size of the worm stays highly variable even though the number of segments is now constant (Moment, 1946) . See Figure I.4A

The mechanism of segment number regulation during regeneration is different from the mechanism during embryogenesis. Even though the segments are still generated antero-posteriorly in a counting-machine manner, the mechanism is different. One could easily imagine that once the body is constructed (i.e. after the embryonic stages), each region of the body acquires its own positional information. The positional information instructs the cells that they have a potential to regenerate dependent on the segment they come from. Since it is probable that the cells generating the new segments come from the most posterior segment left after ablation, this

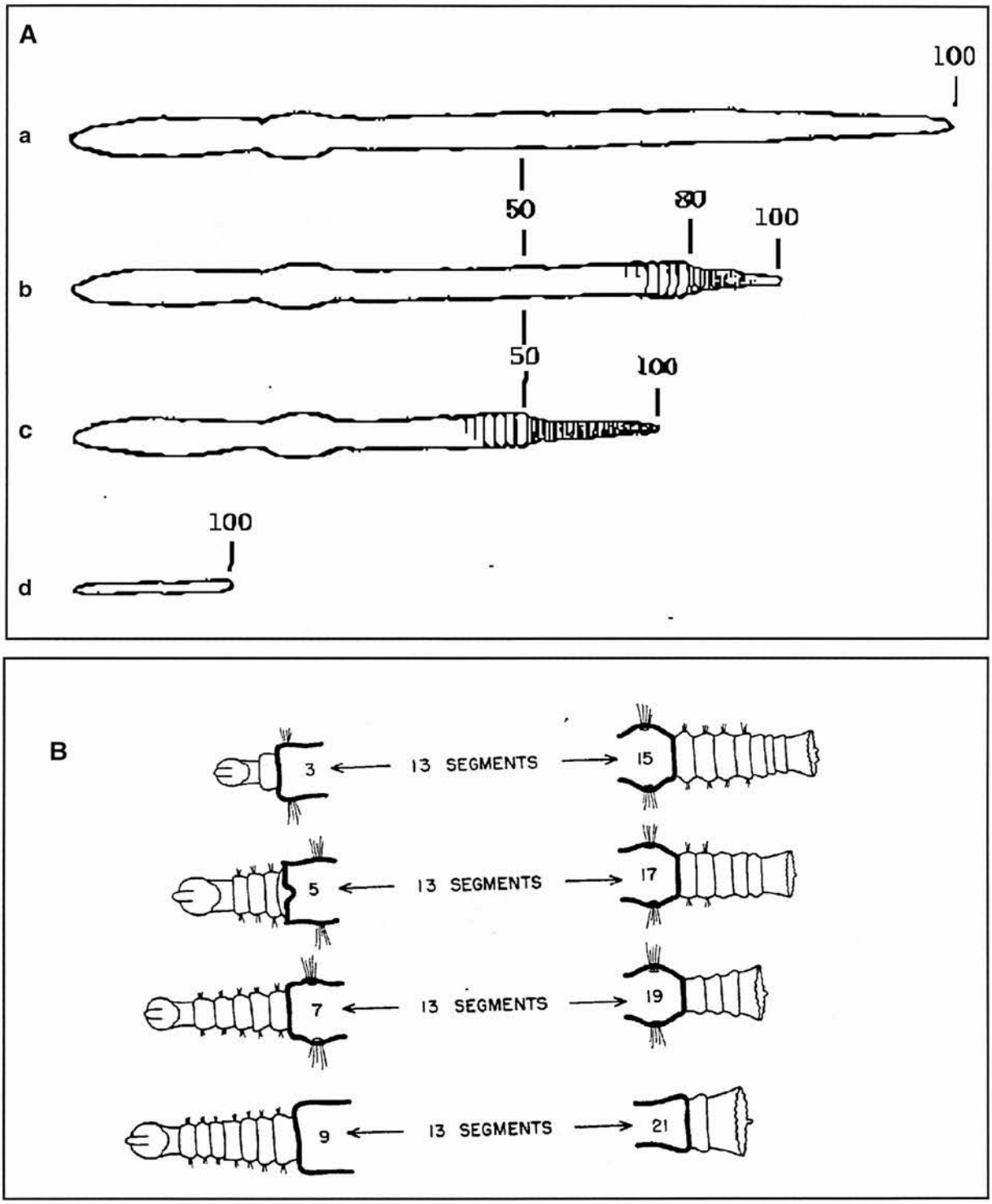


Figure I.4 Two examples of annelids which regenerate an exact number of segments. (A): Eisina foetida regenerates segments until the total number is 100 regardless of the size of the animal. (a) adult with 100 segments, (b) condition at the end of proliferation after amputation at segment 80, (c) after amputation at segment 50, (d) young worm with full complement of segments. (B): Simultaneus anterior and posterior regeneration from pieces 13 segments long cut from 4 levels ofClymenella torquata.

could explain the regular number of segments in regenerated embryos: the most posterior segment has the positional information and potential to regenerate the specific number required to reach 100 segments.

Clymenella torquata, a Polychaete with a precise number of segments

The Polychaetes are not as closely related to the leech as the Oligochaetes are, but they develop roughly in the same manner. The Malanid worms possess an exact number of segments, characteristic of their species, and *C. torquata* possess exactly 22 segments. The family of Malanid worms is extraordinary in that it can regenerate segments cut anteriorly and posteriorly. But most extraordinary, the regenerated segments correspond exactly to the segments that were ablated. This suggests that the polarity and the positional information within the ablated worm is conserved (Moment, 1951) Figure I.4B. Possibly, then, the mechanism of regulation of new segments is the same as in the earthworms: once the whole body is formed, each segment has its own positional information, which endows it with the potential to regenerate a number of segments proportional to its rank.

Segmentation in Drosophila

A summary of *Drosophila* development can be found in Figure I.5 (Wieschaus and Nüsslein-Volhard, 1986). The *Drosophila* is a long germ band insect, that is the germ anlage covers most of the blastoderm and its fate map corresponds directly to the early embryo. After fertilisation, the nuclei divide many times without cell division, creating a syncitium (syncitial blastoderm) where molecules can diffuse freely to act upon the nuclei. The rapid nuclear divisions mean that the embryo relies almost entirely on maternal transcripts for the early development. After cellularisation (cellular blastoderm), cell division continues more slowly. Segmentation is visible very soon after cellularisation, 1h after the onset of

Figure I.5 Embryonic stages of the *Drosophila melanogaster* embyo (after Wieschaus and Nüsslein-Volhard, 1986).
with approximate timing at 22° C

Stage 1: freshly layed egg (0 to 15 min)
Stage 2: early cleavage (15 min to 1 h 20 min)
Stage 3 : pole cell formation (1 h 20 min to 1 h 30 min)
Stage 4: syncitial blastoderm (1 h 30 min to 2 h 30 min)
Stage 5: cell formation, cellular blastoderm (2 h 30 to 3 h 15 min)
Stage 6: early gastrulation, ventral furrow formation (3 h 15 min to 3 h 35 min)
Stage 7: midgut invaginations (3h 35 min to 3h 45 min)
Stage 8: germ band extension (3 h 45 min to 4 h 30 min)
Stage 9: stomodeal plate formation (4 h 30 min to 5 h 10 min)
Stage 10: stomodeal invagination (5 h 10 min to 6 h 50 min)
Stage 11: three-layered germ band (6 h 50 min to 9 h)
Stage 12: shortening of germ band (9 h to 10 h 30 min)
Stage 13: shortened embryo (10 h 30 min to 11 h 30 min)
Stage 14: head involution and dorsal closure (11 h 30 min to 13 h)
Stage 15: dorsal closure complete (13 h to 15 h)
Stage 16: condensation of CNS (15 h to the completion of embryonic development)

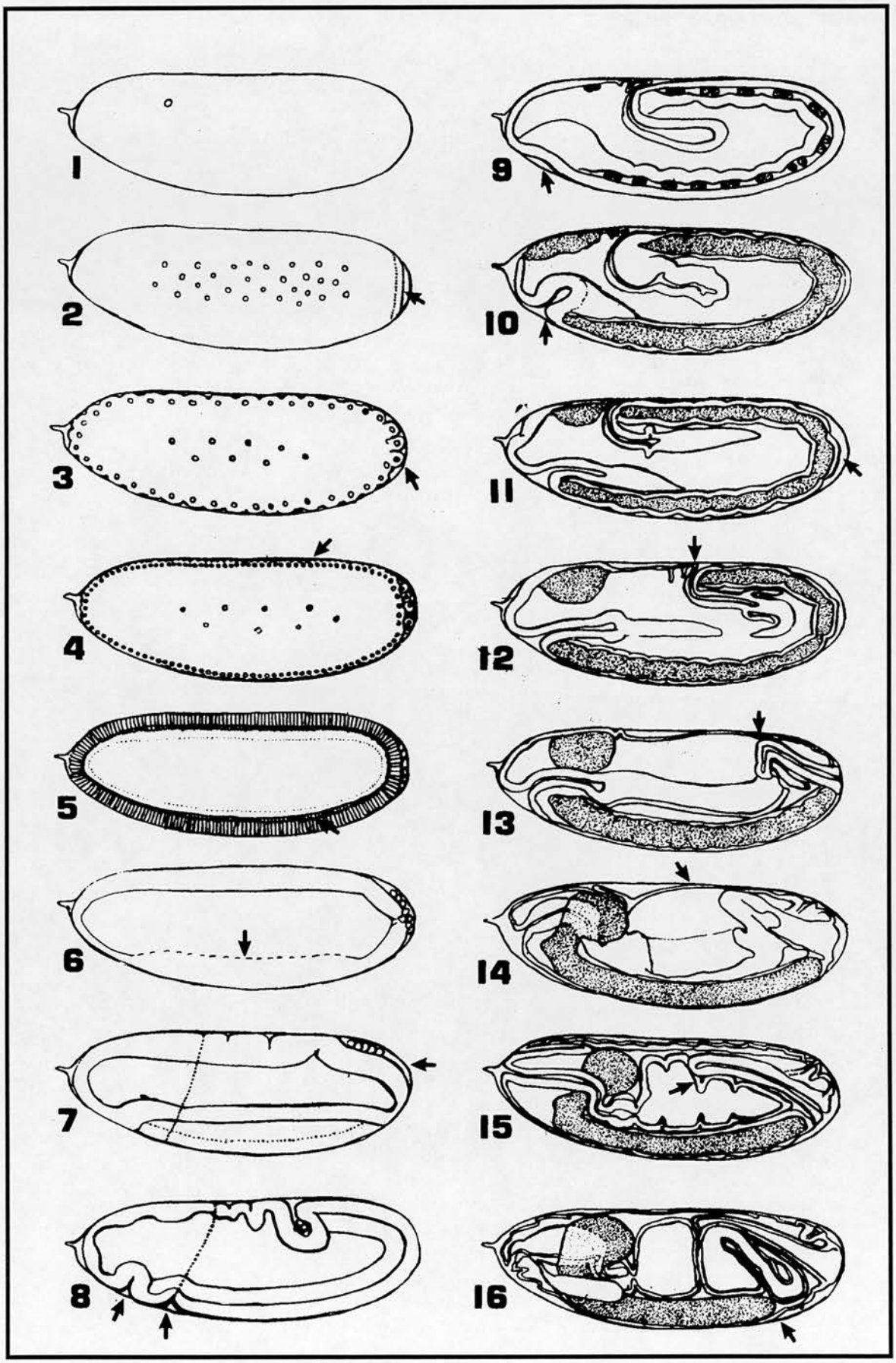


Figure I.5

gastrulation, as a repeated pattern of bulges in the ventral ectoderm[1] (Nüsslein-Volhard and Wieschaus, 1980). The number of bulges corresponds to the number of segments in the differentiated embryo. At germ band retraction, all the segmentation genes have started being expressed (see Figure I. 6). The segments appear more or less all at once, in the mesoderm first, then in the ectoderm, and cover the whole blastoderm.

Much has been learned in *Drosophila* from observation of mutants: it is from such observation that the hierarchy of segmentation genes was determined, (Nüsslein-Volhard & Wieschaus, 1980). The study of mutants allows for models of development to be postulated: mutants show the range of patterns available by modifying only one gene, and where the developmental constraints lie (i.e. which patterns are never possible).

At the outset of development, egg-polarity genes (or cardinal genes, or genes of the maternal system) define the spatial coordinates of the embryo. Three systems, the Anterior, Posterior and Terminal systems are responsible for the patterning of the whole body, respectively the head and thorax, the abdomen, and the acron and telson. Mutation in these genes cause not only the absence of structures specific to the areas they pattern but also absence of the whole part of the body they normally pattern (St Johnston and Nüsslein-Volhard, 1992). The product of 2 of these genes, the bicoid protein (from the Anterior system) and nanos protein (from the Posterior system) are inhomogeneously distributed in a gradient at the anterior pole and the posterior pole respectively. It is the distribution of these two genes independently that is responsible for the proper expression of the downstream segmentation genes. In effect, the product of the cardinal genes control the size of the embryo at the same time as they control

[1]The groves that appear in the outer surface of the ectoderm do not demarcate future segments but parasegments. These are metameric unites which include cells in the posterior part of one segment and the anterior part of the next. Since all segmentation genes respect parasegmental boundaries, it has been suggested that the parasegments are therefore the real segmental unit (Martinez-Ariaz and Lawrence, 1985).

Figure I.6 Morphology of stages during development and corresponding gene activity established at each of these stages (after Akam, 1987)

(A) Pole cell formation, migration of the nuclei to the periphery of the egg. Determinants are localised at the poles of the egg: *bicoid* transcripts are localised at the anterior pole (crosses) and *oskar* transcripts are locallised at the posterior pole (dots); as a result of the transcript locallisation, the *bicoid* and *oskar* proteins follow a graded antero-posterior gradient. These are two of the many maternal genes locallised at the time of laying. These in turn control the domain of expression of the gap genes, such as *hunchback* (in gray), into a graded distrbution.
(B) Syncitial blastoderm, the nuclei reach the perimeter of the egg and become transcriptionally active. Localised transcription of the gap genes *hunchback* and then *Krüppel* (sharp dark band) is established from the zygotic genome, under the control of the maternal genes. For example *hunchback* is activated and *Krüppel* is repressed by high levels of bicoid protein.
(C) Cellular blastoderm, cell membranes are being formed but cells are not yet closed. The expression of pair-rule genes under the control of the gap proteins, resolves in to a well-defined pattern of overlapping stripes showing double segment periodicity.
(D) Gastrulation. Mesoderm invaginates ventrally; anterior and posterior midgut invaginations for m the endoderm. The expression of engrailed (dark grey stripes) and other segment polarity genes (under the control of gap proteins and pair-rule proteins) appears in segment periodicity.
(E) After germ band extension, during stomodeal formation, the metameric region forms a uniform double-layered structure extending around the posterior pole of the egg, and the most posterior segments are apposed to the head. Cell division is underway, most blastoderm cells undergoing 2 or 3 rounds of mitosis before differentiating. Cells expressing *engrailed* (dark grey) and *wingless* (light grey) define the definitive metameric pattern and flank presumptive parasegment borders.

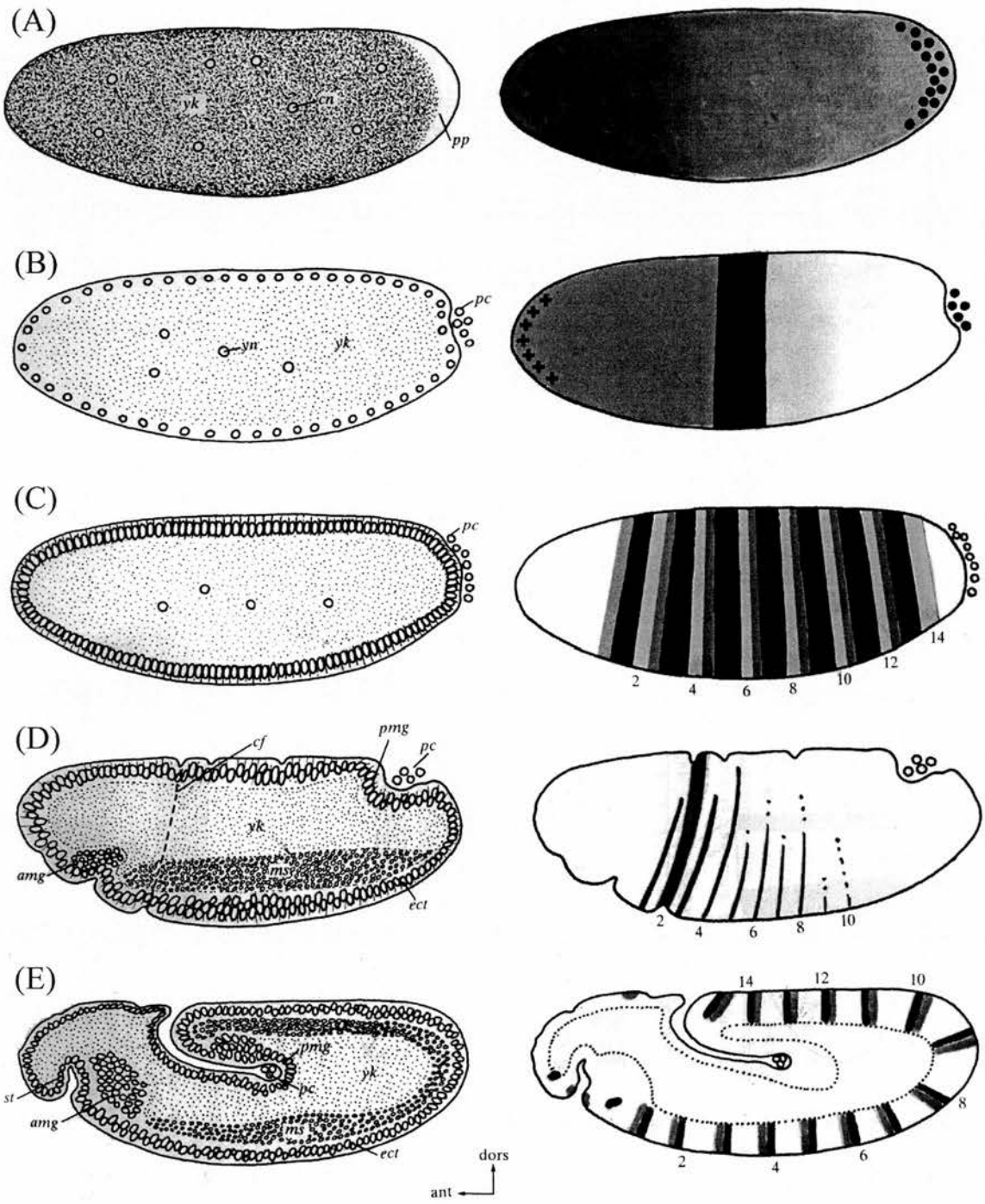

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Figure I.6

regional specification: the establishment of segmental primordia in the blastoderm depends first on the proper establishment of these maternal genes.

As a result of maternal gene expression, gap gene transcripts (such as *Krüppel, hunchback, knirps, giant*...) appear at the syncitial blastoderm, in broad sections of the embryo correlating with the distribution of bicoid and nanos proteins. Mutants in these genes result in a gap in the pattern of the body plan. In Krüppel, runt and knirps mutants, the defect is obvious 15 min after the onset of gastrulation. All 3 mutations cause reduced germ band, prefiguring the reduced number of segments observed in the differentiated larva. Again, like in cardinal gene mutants, a part of the body is deleted.

Pair-rule genes (such as *hairy, even-skipped, fushi-tarazu*...; see Chapter IV for more details) appear after the gap genes, translating the smooth wide expression of gap genes into 7 sharp stripes of pair-rule gene expression. The pair-rule genes are the first overt sign of segmentation, and presumably the first genes to create boundaries (Lawrence, 1987). What is most surprising in the building up of segments in *Drosophila* is that the resulting segments are not actually homologous. Each segment results from a different history of gene regulation and expression, as best examplified in the transition from gap gene expression to pair-rule gene expression: 7 stripes within the embryo might look similar, but each is actually the result of different gene interactions.

Finally, the segment-polarity genes (such as *engrailed, wingless, patched, hedgehog*....; see Chapter V for more details) appear in 14 stripes, reflecting the adult periodicity. The order in which these genes appear reflect partially how one class of genes is required for the proper expression of genes the next class (for more detail, see Akam, 1987 and Ingham, 1988). What is most striking is the way in which smaller and smaller domains of

expression are specified at each step. It has been argued that with such a small number of cells at the time segmentation starts, a map cannot be very precise: the larval body is comprised of 3 thoracic and 8 abdominal segments, but at the blastoderm stage, the epidermal primordium giving rise to thorax and abdomen is only about 40 cells long. An initial subdivision into double segments avoids problems of accuracy that would be encountered in the simultaneous establishment of segment boundaries every 3 to 4 cells. Therefore the segmental pattern starts with a crude map, which is gradually refined as the embryo develops and the number of cells increases.

The other genes that start to be expressed as a result of the expression of the segmentation genes are the Homeotic genes, or segment identity genes. These genes are not directly correlated to segmentation but are responsible for regional specification of the whole body. The boundaries of the regional pattern always corresponds with the expression of the segmentation genes , and spans the same segments e.g. the product of the homeotic gene *Ultrabithorax* (*Ubx*) always spans parasegments 5 to 13 exactly (Beachy, et al., 1985). Mutations in the maternal or gap genes result in a similar shifting of expression of the segment polarity genes and the homeotic genes.

The mutants isolated in *Drosophila* therefore establish that the number of segments depends on the correct expression of a limited number of genes. A modification in the number of segments is always correlated with the ablation of a part of the body. Even in instances where the "grain" of the pattern is altered, by experimentally modifying the number of cells in the embryos (Busturia and Lawrence, 1994), there is no modification of the number of segments. Instead, there is regulation by modification of the number of cells per segment.

From these experiments, it seems that *Drosophila* fits the French Flag model of pattern formation. The whole field to be segmented is present at the time segmentation start, and there is evidence that it is being "measured" at the onset of segmentation. Meinhardt (1988) has suggested a means by which 2 gradients, whose sources are at the opposite ends of a field, could cooperate to set the size of the field. The bicoid and nanos protein gradients however do not interact, and each one is set up independently, delimiting their own field for further segmentation. It is possible that the size of the embryo affects the slope of the bicoid gradient, thereby affecting the position of the thresholds (see figure I.1 A). This would fit with the observed fact that the absolute size of the segmental field depends on the absolute size of the embryo, and the field is being subdivided into smaller fields proportionally.

Since the number of segments in *Drosophila* seems correlated to the expression of segmentation genes, is it possible to find in related species (if not in mutants), a modification of the number of stipes of expression of segmentation gene corresponding to a modified number of segments? In the beetle, pair-rule genes are expressed in 8 stripes instead of 7 in *Drosophila*, and their number of abdominal segments is also increased from 8 in *Drosophila* to 10 in the beetle. Understanding of how the beetle got its eighth stripe might help us start understanding what limits the nmber of segments in *Drosophila* and other insects with a limited number of segments

Segmentation in the other insects

The entire body plan of the *Drosophila* embryo is already established by the blastoderm stage. This is the case in all long germ band insects including other Dipterans. For the majority of other insects, namely the short germ-band and the intermediate germ-band insects, however, development occurs in a different way. In short germ-band insects, a syncitial and cellular blastoderm is also formed, but only a small fraction of

the blastoderm (the germ anlage) contributes to the embryo. The remaining blastoderm gives rise to extra-embryonic membranes. Furthermore, the entire body plan is not yet established in the syncitial blastoderm: only the head region is specified. All the more posterior regions of the embryo are generated by a posterior growth zone after gastrulation and cellularisation. In intermediate germ-band insects, the head and the thorax are established in the germ anlage, and the rest of the embryo is generated by a posterior growth zone (French, 1990; Patel, 1994).

These differences in early development have many different consequences for the way the body plan is established. Mainly, there can be no proportional subdivision of the body plan in regions as in *Drosophila* because (i) not all the body axis is present from the time segmentation starts, therefore there is no way of measuring it in the same way as in Drosophia with the Anterior, Posterior and Terminal systems, and (ii) diffusion of molecules such as bicoid or nanos would not be possible because the body is cellularised at the time segments are being set up: diffusion of such large molecules would be slowed down by the cell membranes, rendering the patterning difficult or even impossible in the time required. In the case of these insects, it could be possible that patterning happens first in the germ anlage in a French Flag manner, setting such things as the potential size of the embryo, and that segmentation then proceeds as a Counting Machine.

It is interesting to note that the way the different types of insect segment is not correlated with their phylogenetic relationship, that is, short, intermediate and long germ-band insects are found dispersed in the different families of insects. This suggests that the phylotypic stage towards which they all converge, the complete segmentation, is really a constraining step of development.

Segmentation in vertebrates

The main segmented structure in the vertebrates are the somites, which give rise to the vertebrae and are also responsible for the segmentation in the neural tube (and neural crest derived structures such as the dorsal root ganglia). There are many approaches to describing segmentation of the somitic mesoderm in the vertebrates: following the lineage of cells (Eisenberg, et al., 1992; Holland, et al., 1992), looking in the extracellular matrix for changes of properties that may account for aggregation of the cells (Bellairs, 1979; Bellairs et al., 1978) or studying patterns of homeotic gene expression (Krumlauf, 1992; Krumlauf, 1994). Data from all these different approaches are required for understanding the mechanism of segmentation and eventually how the number of segments is regulated.

Somites form by sequential epithelialisation of two mesenchymal rods of mesoderm, the segmental plates, which are laid down during gastrulation. In higher vertebrates, these appear on each side of the midline neural epithelium as the primitive streak regresses along the antero-posterior axis of the embryo (Figure I.7; Keynes and Stern, 1988). In the chick, at the primitive streak stage, the somite-forming cells come from Hensen's node and from a region caudal to the node (Selleck and Stern, 1991). As the node regresses, it lays down cells that populate the segmental plates and eventually contribute to the segments. Somites form by arranging themselves into an epithelial sphere with all the cell-cell associations of an epithelium. The grouping of the cells is probably initiated by a differential expression of cell adhesion molecules: the cells in the somites are more adhesive than that of the segmental plate (Bellairs et al., 1978).

Segmentation in some lower vertebrates such as Xenopus and zebrafish show dissimilarities from the vertebrate general mechanism of somite formation (Hamilton, 1969). Namely, segmentation happens after

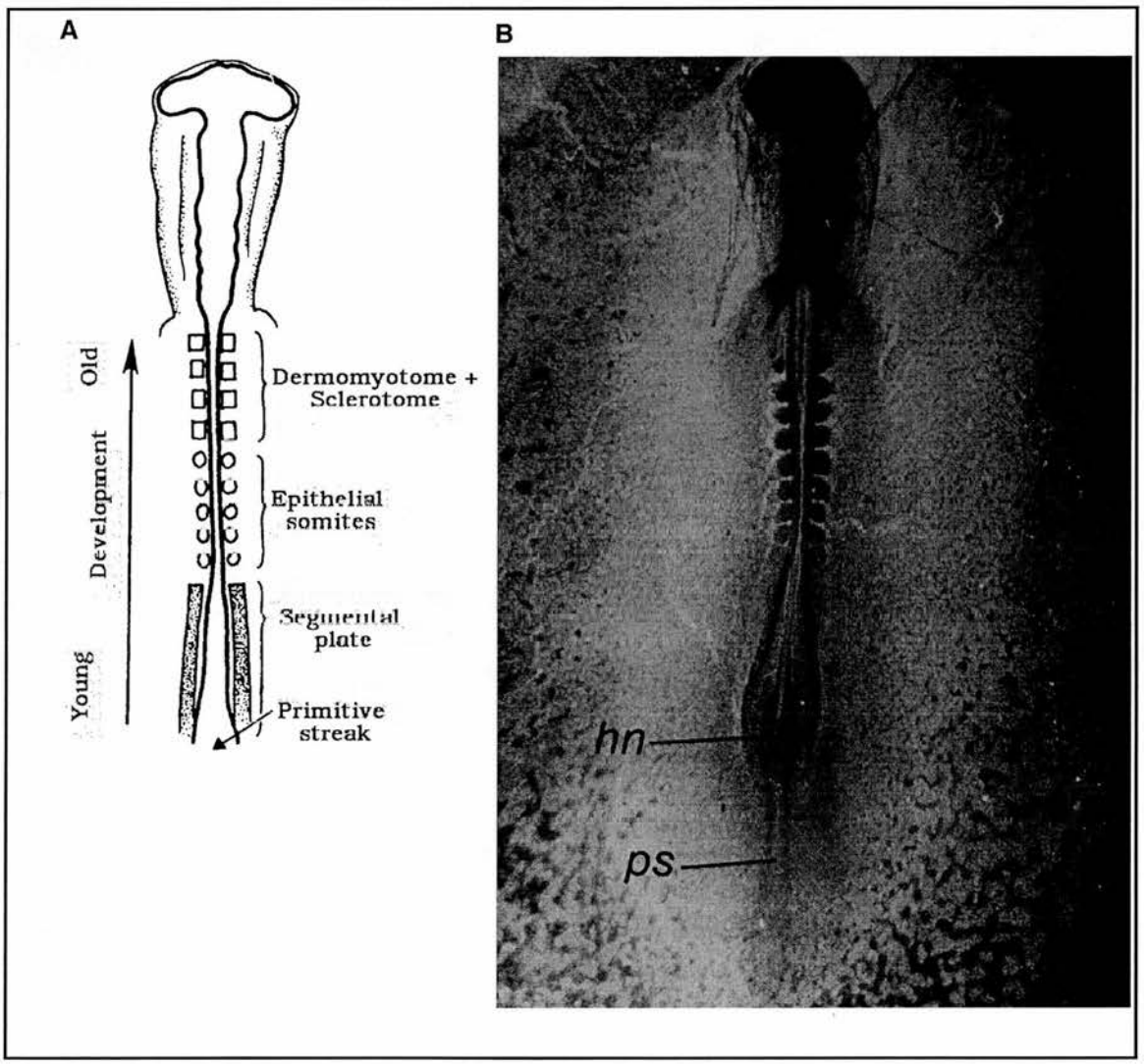


Figure I.7 (A) Diagram of the main stages of somite formation. Somitogenic cells arise towards the posterior end of the embryo (bottom of the diagram) and remain in the segmental plate until they segment into epithelial somites, in anteroposterior sequence. **(B)** Whole mount of 8-somite chick embryo. The neural tube opposit the segmental plate is still open, and the notochord can be seen between the elevated neural folds at this level. More posteriorly, Hensen's node (hn) is clearly visible, as is the primitive streak (ps) (From Keynes and Stern, 1988).

gastrulation has proceeded. The presomitic mesoderm is laid down during gastrulation, and the differentiation into somites follows an anteroposterior spatiotemporal gradient. The other difference is that the somites of *Xenopus* do not form such an organised epithelium , and lack the large interconnected networks (gap junctions and tight junctions) typical of a mature epithelium. This might affect communication within the field and therefore limit the number of possible mechanisms of segment regulation.

Determination of the number of somites

In wild-type embryos, in a normal environment, (i) the size of the animals is variable, (ii) the number of segments is constant (Maynard Smith, 1960), and therefore (iii) the somite size varies proportionally to the whole embryo size. A different way of linking these 3 observations is to say that (i) the size of the animals is variable, (ii) the somite size varies proportionally to the whole embryo size, and therefore (iii) the number of segments is kept constant. The difference between these two interpretations reflect two different mechanisms of pattern formation. In the first case, segmentation is global, i.e. segment size is constrained by the total size of the segmental field. In the second case, segmentation is local, i.e. the segment size is constrained by the size of the building blocks (e.g. cells) of the whole embryo.

Experimental animals or mutants where the size of the cells is modified, or where the number of cells is modified can be observed to help us distinguish between the two hypothesis. I will be describing both types of experiments.

In *Xenopus*, Hamilton (1969) noticed that haploid animals have a body size comparable to that of diploids, but with smaller cells. In those embryos, the number of somites is constant; i.e. the number of cells per somite is increased (compared to the diploids) to produce the same number of somites. In mouse, tetraploidy leads to embryos having larger cells than

the diploids (about twice the size), and a slightly smaller body (10% shorter). In this case, cell density is reduced (by approximately 50%) and the "grain" of the developmental map is reduced (Henery, et al., 1992). This causes some of the tissues with "fine grain" such as the brain to be distorted, but no anomaly is found in the somites, presumably containing fewer cells than normal embryos. Both these experiments suggest that the size of the cells is not what determines the size of the embryo : segmentation is subject to global rather than local regulation. The number of cells is not a constraint on the segmental pattern.

Modification of the number of cells was done experimentally in *Xenopus* by removing or adding part of the pre-segmental tissue. Removal of segmental tissue (Cooke, 1975) or addition of tissue (Waddington, 1938), before gastrulation and somite formation, produces embryos with a normal number of somites. In these cases, the somites initially contain smaller or larger number of cells to keep in proportion with the modified segmental tissue size. The resulting embryos are normal except for their modified size. Mouse offers the advantage of allowing the use of mutants as an alternative to manipulation where the environment might be pertubed. The *amputated* mutant is smaller than the wild type, due to a smaller number of cells. Again, the number of somites observed in these mutants is the same as in wild type (Flint, et al., 1978). The number of cells in the somites is initially smaller, such that the size of the somites is proportional to the size of the body. Flint and colleagues suggest that regulation happens by pre-somites probing for the size of pre-somitic mesoderm (which he describes as a morphogenetic field), that being reduced too in the mutant. These experiments varying the number of cells in the segmental field (or the pre-somitic mesoderm) are consistent with the experiments reported above of varying the size of the cells: the number of segments, and not the cell size or number, seems to be a developmental constraint, specific to each species.

In the view of these experiments, it is probable that regulation of somite size is achieved by assaying body size rather than by counting cells. It is interesting to note that in similar experiments done in *Drosophila*, the number of segments was also unaltered: embryos made to carry 7 copies of the bicoid gene have an abnormally small number of cells (diminished by 35%) in the gastrula. Although patterning within the segments is altered, the number of segments itself is unaffected (Busturia & Lawrence, 1994).

Lineage tracing, cell transplantation and the heat-shock experiments

The previous paragraph was concerned with finding out how the pattern of segmentation is determined. In this paragraph, I will review what we know about where the cells that populate the segments come from.

Lineage tracing has brought different information on the mechanism of somite formation in different species. In the chick, it is now kown that the somites cells come from Hensen's node as well as from a region caudal to the node. Single cell lineage have shown that cells from the node lay down cells at periodic intervals to populate segments (Selleck & Stern, 1991). In the zebrafish, clones deriving from a single gastrula cell populate muscles exclusively, also with a periodic distribution, although different embryos exhibit different periods (Kimmel and Warga, 1986). This suggests that there might exist segment founder cells responsible for either forming the part of a somite or generating a nucleus of cells to recruit neighbouring cells into the developing somite. These founder cells however, at least in the chick, are not commited at the time they are in the node: transplanting cells from the node into the notochord makes them follow a notochord fate (Selleck and Stern, 1992). Also, segmentation preceeds normally and a normal number of segments is formed when the node is extirpated (Bellairs, 1986; Bellairs and Veini, 1980). This suggest that if the segment founder cells exist, they might require some external cues for segmentation.

Experiments on amphibian embryos have provided evidence that heat shock causes a disruption of segmentation visible several hours after treatment (Elsdale, et al., 1976). This disruption generally consists of fusion of somites together. Disruption by heat-shock has since also been observed in other vertebrates such as the chick (Primett et al., 1989; Primett et al., 1988) and the zebrafish (Kimmel et al., 1988; Kimmel and Warga, 1987). It has been established that heat shock affects the cell cycle (Primett et al., 1989). How does the disruption of cell cycle affect a specific somite or a specific group of somites? It has been suggested that the cells destined to segment together have a degree of cell synchrony, thereby all the cells destined to form the next segment, and only those, are affected by heat shock. This is consistent with the fact that the effect of heat shock is not seen in the somites forming when the heat-shock is delivered, but in those that begin to form some hours later. Indeed, the time between the heatshock and the first defect has been established in chick at least, the be corresponding to the length of exactly one cell cycle, and the period of heat-shock sensitivity moves at the same period as somite formation (Primett et al., 1989).

However, vertebrate embryos vary in their response to heat-shock. A summary of the main differences is presented in Table I.2. Thes differences of phenotype reflect striking differences in somite formation. The most important difference between the lower vertebrate models (Xenopus and zebrafish) and the higher vertebrate model (chick) concerns the number of defects resulting from one heat shock. Although in chick several defects are found in a periodic fashion, in Xenopus and in zebrafish, only one defect is found. This has strong implications in terms of mechanism of segmentation (see below, "models of vertebrate segmentation")).

	Morphological defect	*Time between h.s. and first visible defect*	*First affected somite*	*Rate of somite formation*	*Period of h.s. sensitivity*	*Periodicity*
Chick[1,2]	larger or smaller somite, or fusion of 2 consecutive somites.	9-10h (=length of 1 cell cycle)	6-7 somites	1 somite per 1.5h	Neurulation (early somites formation)	2 or 3 defects may be observed, all separated by 6 or 7 somites.
Xenopus (early)[3,4]	missing intersomitic fissures; rudimentary or branching fissures; abnormal organisation of somitic cells; modification of the orientation of the myoblasts. Stretches more than 1 somite.	4.5 h	Depends on stage at time of h.s. Varies from 8 to 4 somites	Varies from approximately 2 somite per h to 1 somite per h	Neurula stage: from stage 12 to stage 25 (visible segmentation starts at stage 17)	None: only one defect, affecting a variable number of somites in a stretch. The number of somites affected depends on the length of the h.s.
Xenopus (late) [3,5]	modified timing and positioning of fissure. Stretches more than 1 somite.	2.5 to 24h	random	Varies from approximately 2 somite per h to 1 somite per h	Pre-neurula stage	None: one patch is affected, with normal somites anteriorly and posteriorly.
Zebrafish [6,7]	Borders of myotomes are displaced, muscle fibers are too short or too long and sometimes not correctly aligned. Stretches more than 1 somite.	2 to 2.5 h	4 to 5 somites	2 somites per hour	Post-gastrula embryo (from begining of segmentation)	None: defect is observed in only one stretch, in several somites.

Table I.2 Effect of heat-shock (h.s.) on different vertebrate models;1. Primett et al., 1988; 2. Primett et al., 1989; 3. Elsdale et al., 1976; 4. Cooke, 1978; 5. Elsdale & Davidson, 1986; 6. Kimmel & Warga, 1987; 7. Kimmel et al., 1988.

Does the control of the number of somites come from the tail of the chicken?

In chick (amniote), the posterior boundary of the embryo is not yet established until many of the somites have formed (Bellairs, 1986). It is therefore impossible for size regulation to happen by subdivision of the body plan. At most it could be argued that there is a measuring of the pre-somitic mesodermal plate going on (pre-pattern model, see below). But this does not explain the total regulation of the number of somites. Another explanation for regulation came from the observation that there is more potential pre-somitic tissue being formed than is actually required, as intensive cell death can be observed in the tail bud, where segmentation ceases (Bellairs, 1986). It could be that the chick controls the number of segment at the stage when the posterior boundary is formed, by deleting extra potential somitic tissue. This does not resolve the question of how the embryo knows how many potential segments to cut off, but merely points in the direction of where the regulation might come from. It is possible that a gradient is set up towards the end of segmentation, along the antero-posterior axis, for it to be "measured". As we have seen, there is evidence that the size of the somites is proportional to the size of the body. So, in order to make the right number of somite, the embryo only has to produce a body of the right size. The "measure" then does not need to be integrated into a number of segments.

The models of vertebrate segmentation

In Xenopus, it seems possible that there is measurement of the total pre-somitic mesoderm length, rather like in *Drosophila* where the bicoid-nanos system might be measuring the lenght of the embryo before subdivision. In chick, this is not possible, since all the precursors of the somites are not present when the first one is layed down.

On the basis of the observations and experiments described previously, models of segmentation have been proposed. The model of pre-pattern has been advanced for explaining somite formation in the chick : this suggests that the segmental pattern is present in the segmental plate . However, The model suggests that the pattern is formed locally in the segmental plate. We have seen that there is evidence that segmentation must be global rather ran local in order to regulate the size of the somite to the size of the whole body. However, the models remains to be tested. A model of positional information was proposed by Meinhardt(Meinhardt, 1986), but again this deals with local pattern formation, suggesting how the pattern within the somites might be generated. By dealing with the local formation of somites, these models cannot deal with the problem of regulation of segment number.

The only global model of segmentation so far, is the Clock and Wavefront model advanced by Cooke and Zeeman (Cooke and Zeeman, 1976). This model involves a clock (the cell state, for example the cell cycle), acting like an intracellular oscillator, and a wave of determination (somitogenic cell determination). The wave travels antero-posteriorly, and when its peak hits a group of cells (synchronous), they undergo somitic differentiation only if they are in the appropriate state.The total number of somites will be unaffected by any variation in embryo size if both the clock and the wave speed are correlated. Heat-shock experiments in Xenopus do fit this model: a heat -shock in Xenopus results in only one, delayed, defect in the segmental pattern. The cell cyle can be compared with an oscillator (or clock), while the wavefront (or propagating wave) would periodically pick among the cells those in the right state of oscillation to differentiate and go on to form a somite. The cells that will go on to form somites are determined by their synchrony, and therefore a heat-shock affecting the cell cycle will affect precisely the cells deteremined to segment together. In

chick, where heat-shock gives rise to a number of defects in a repeated pattern (Primett et al., 1988). the model would require adjusting: one wave doesn't determine only one segment anymore but several.

The differences in the setting up of segmentation in the different vertebrates has made it impossible so far to form a theoretical model that would encompass all the differences seen between the different embryos. It could be argued that some of the animal models used are not representative of the vertebrates in general (Bolker, 1995) . This might be true but then the evolutionary distance between the different vertebrates makes it possible that all modern vertebrates derive from a common segmented animal. Modern vertebrates vary from the ancestral form of segmentation, and it is possible that they have kept part of the basic mechanism. However, finding out what the ancestral mode of segmentation was might not be an easy task.

In this phylum like among invertebrates, some embryos could be measuring the total length before subdivision, whereas in others, the posterior limit of the segmental tissue is not determined when the somites start forming, requiering a different method of control. What this means for the study of segmentation is that there are general points that might be re-used in different segmental systems, even if they did not originate from a common segmental system.

The aim of this thesis

This thesis sets out to examin how segment number is regulated in the leech embryo. There are currently no models for this. Faced with so many different means of regulating segment number and segment size, it is impossible to assert definitively which group the leech fits in best. On first approach, it seems that the leech might ressemble what happens in the chick, with posterior destruction of extra tissue otherwise fit to segment.

The setting up of segments in the leech is examined here by means of observation of the cells involved and by searching for molecular markers.

On the basis of these observations, I attempt a model fitting the data found in the leech. The model might be only true for the leech, or might throw some light on the mechanism involved in other systems. In effect, evolutionary convergence in the mechanisms of segmentation seems to be frequent, maybe due to the fact that segmentation is the result of developmental constraints common to all or many of the segmented species. Therefore, looking for a mechanism of regulation in the leech may well be a legitimate way to learn how some vertebrates control their segment number!

Chapter II: A description of the supernumerary cells

Introduction

The leeches (Hirudines family) are annelids closely related to the Oligochaetes, such as the earthworm (*Eisina foetida*). As the Oligochaetes, they are segmented animals, but they differ from this close family in the fact that they have an exact number of segments (Whitman, 1892). Oligochaetes such as earthworms (*Eisina foetida*) have an average number of segments (Moment, 1946) and a capacity to regenerate lost segments (Morgan, 1901). An exact number of segments is found in other species of annelids, among the Malanid polychete worms(Moment, 1951). It is interesting that the leech is more closely related to the Oligochaetes (with a less exact number of segments) than to the Polychaetes (with an exact number of segments), confirming the idea that the mechanisms leading to the phylotypic stage can be quite variable (see Chapter I).

However, the Oligochaetes and the leeches are closely related and their development is very similar (Storey, 1989a), with teloblasts dividing in a stem-cell manner to give rise to a chain of blast cells. It is therefore possible that the mechanism regulating the number of segments in the leech was acquired through very few evolutionary steps since the species diverged from the common ancestor of Hirudines and Oligochaetes. If this is the case, the change might rely on a small number of molecules, recently acquired or modified.

In this chapter, I address the question of the regulation of the number of segments in the Glossiphoniid leech *Helobdella robusta*, by the observation and characterisation of the most posterior cells produced by the teloblasts: the most posterior blast cells, even though they are produced by the teloblast in the same way as the more anterior ones, do not give rise to any of the segmental body but reportedly degenerate (Fernández and Stent,

1980; Shankland, 1984; Torrence et al., 1989; Weisblat et al., 1984; Zackson, 1984; Zackson, 1982). In this study, I also attempt to characterise the type of cell death these cells undergo, in order to relate the observations to known mechanisms, at the molecular level.

Setting up of segments in the leech

See also Chapter I for general leech development; Figure I.2 and Table I.1 for a description of the stages.

As an annelid, the leech develops through spiral cleavage albeit modified. (Sandig and Dohle, 1988) It follows a highly stereotypic and autonomous development, especially in the early stages. By the second cycle of cell division, leading to the cells A, B, C and D, there is already segregation of the cell responsible for the generation of the whole of the segmental tissue, the D cell.

With a few more divisions of the D cell, 10 teloblasts are born. These cells are born at slightly different times, starting with the birth of the M teloblasts about 13 h after egg deposition, and ending with the birth of the O/P teloblasts 22 to 28h after egg deposition (Bissen and Weisblat, 1989). The timing was recorded in *Helobdella triserialis* at 25°C and the development of *H. robusta* is very similar, with the timing approximately the same (see Table I.1).

Each of these teloblasts acts in a stem-cell manner, producing blast cells by dividing regularly at the approximate rate of 1 cell division every 1h20 min (Zackson, 1982). As the blast cells of each lineage are produced, they form a line, chain, or bandlet of blast cells, in an antero-posterior age gradient. Each of the blast cells goes on to divide and produce a clone of cells to populate part of one or several segments, depending on their lineage. Cell lineage studies have revealed that all the blast cells from the same teloblast divide in the same way, always populating the same part of the segment(s). There are two types of teloblasts, monomeric (M, O and P) and dimeric (N and Q), i.e. giving one or two blast cell(s) per segment

(Shankland, et al., 1991). In the N and the Q lineages, the teloblasts produce two types of blast cells in turn. In these two lineages only, one segmental complement descends from two primary blast cells. In the other lineages, one primary blast cell is at the origin of one segmental complement. The different segments do not vary much from the common ground plan. One exception comes in the midbody segments 5 and 6, the reproductive segments. Here, some of the blast cell descendants follow a different fate than in the other segments, making specialised structures, found only in these two differentiated segments. Later in development, the most anterior segments fuse to make the head, and the most posterior fuse to make the tail, reducing the number of ganglia to 23 in the adult.

The teloblasts do not produce only segmental tissue: they also produce some of the tissue for the head and some supernumerary cells, i.e. blast cells at the very posterior end that do not give rise to further tissue. The teloblasts themselves are thought to get integrated into the presumptive gut after they have finished dividing (Nardelli-Haefliger & Shankland, 1993).

The other type of cells, the macromeres, are the A''', B''' and C''' cells, which eventually give rise to the endoderm. The endoderm is known to segment (Fernández, 1980; Nardelli-Haefliger & Shankland, 1993), but because segmentation appears later, in a tissue of different origin, and is not synchronous or in register with segmentation in the ectoderm and the mesoderm (Nardelli-Haefliger & Shankland, 1993), it is probably not related to segmentation of these tissues.

In this chapter, only the teloblasts and their descendents directly at the origin of the segmental ectoderm and mesoderm are studied.

The making of the bandlets

The teloblasts divide in a stem-cell like manner, giving at each division one large cell, the teloblast, and one small cell, the blast cell. As

they are born, the blast cells remain posterior to the blast cell produced at the previous cell division, in such a way that all the blast cells form a column or bandlet of cells. They are therefore arranged chronologically, and the bandlet keeps this antero-posterior gradient of development throughout blast cell production.

All the bandlets on each side converge into the germinal band. From this morphological point on, the different lineages are in contact with each other and there is evidence of gap junctions between the cells of one lineage, and those of the other lineages, at least at early stage 7 (Fernández & Stent, 1980; Weisblat et al., 1980). However, the different primary blast cells and their descendants are not yet in register at that time (Lans et al., 1993). At the end of stage 7, the germinal bands from each side meet anteriorly and start coalescing, forming the germinal plate (Fernández & Stent, 1980). From that stage on, segmentation starts being visible, in the form of ganglia and body wall muscles, derived from the blast cells.

Lineage studies have shown that one primary blast cell (two in the case of the N and Q lineage) will divide and populate one hemisegmental complement, and its descendants may span more that one segment (i.e. 2 or 3).

The investigation into the supernumerary cells

Although the leech divides in a stereotypic manner and in an autonomous manner for most of its development, the teloblasts are known to produce more blast cells than required, i.e. more than 32 or 64. The most posterior produced blast cells are reported to die, through an unknown mechanism, and because they do not participate in segmentation, these have been called "supernumerary" cells (Shankland, 1984). The investigation here was to characterise further these special cells, in order to find some insight into the mechanism of regulation of cell number. The study involved lineage tracing to follow these cells late in development, a timing of the cell

death of the supernumerary cells identified, and an attempt at characterising the type of cell death (by various detection methods). This led to the conclusion that the number of supernumerary blast cells is variable from one lineage to another, and even within one individual from one side to the contralateral side in the same lineage. This also led to the suggestion that the cells might not die via apoptosis and traditional programmed cell death. All this points towards a non-autonomous segregation of the "segmental" cells (the segment founder cells) and the "supernumerary" cells (the cells not involved in segmentation).

Material and Methods

Lineage tracing

Helobdella robusta embryos were collected in clutches, and cultured in Htr medium (4.8mM NaCl, 1.2mM KCl, 2mM $MgCl_2$, 8mM $CaCl_2$, 1mM tris base, pH 6.6). In each clutch the embryos were assumed to be synchronous (the onset of development varies over only a few minutes in a clutch, (Fernández, 1980), staged, and then followed through development until the appropriate stage. I recorded the exact time of birth of the relevant teloblast, which was considered the beginning of its blast cell producing cycle (i.e. t= 0h; at t= 1h 20min, the teloblast will have produced 1 blast cell, at t= 2h 40 min, 2 blast cells...)(Bissen & Weisblat, 1989). The relevant teloblast (M, N, Q) or proteloblast (OP) was injected with lineage tracer, either RDA (Tetramethylrhodamine Dextran, MW 10 000; from Molecular Probes D-1817) or FDA (Fluorescein Dextran anionic, MW 10 000; from Molecular Probes D-1820). Both are lysin-fixable, allowing for the sample to be viewed after fixation. The injection procedure was according to David Weisblat's method (Weisblat et al., 1980): RDA or FDA was dissolved at the concentration of 100 mg/ml in 0.1M KCl and frozen in 10µl aliquots. Aliquots were defrosted just before use and mixed 1:1 (v/v) with 2% Fast

Green FCF to a final concentration of 50mg/ml. The mixture was then filtered (Milipore 0.45µm, cat. No UFC0HV25) before injection. Electrodes were pulled on a Sutter Instrument Co. Puller P-87, program: heat 400, pull 150, velocity units, time 125 (heat and time were sometimes slightly modified), using Clark Electromedical Instruments glass GC150F-15. This gave a long tip, suitable for the small cells, but large enough not to get clogged too easity.They were filled with the dye by capillarity. If recordings were made, the electrode was also back-filled with 0.2M KCl with the help of a pulled plastic syringe. The tip was broken by gently pushing the electrode against a glass slide.

To hold the embryos during injection, I used a suction chamber made as follows: at the bottom of a small petri dish (35mm) plastic tubing was glued. A vertical hole was made with a small muscle needle and Sylgard poured over. After the Sylgard was cured, the hole left by the needle was carved to allow the embryo to rest on it while suction was exerted at the other end of the tubing by a syringe pulled by a micro drive.

The chamber was filled with non-divalent medium (4.8mM NaCl, 1.2mM Kcl, 1mM Tris base, pH 6.6) and the embryo was pressure injected, while at the same time, recordings were made of the membrane potential to ensure that the micro electrode was properly inside a cell. This recording was particularly useful for injection of small cells (blast cells or micromeres).

I used a pressure of around 3 and 15 P.S.I. (depending on the tip diameter), for 10ms.

After injection, the embryos were left to recover at 23°C in Htr medium containing gentamycin (40ng/ml). After the appropriate length of time, the embryos were fixed in 4% formaldehyde or paraformaldehyde in phosphate buffer from 1h to overnight. They were then rinsed in PBS and stained with Hoechst 33258 (5µg/ml) for about 1h then rinsed in phosphate buffer saline (PBS). They were stored at 4°C until viewed. They were then

dehydrated through a graded series of ethanol (30%, 50%, 70%, 95%, 100% twice, for 5 minutes each), cleared and mounted in BBBA (Benzyl Benzoate: Benzyl Alcohol 3:2), between slide and coverslip in a well carved in 2 layers of electric tape. This allowed the embryo to be "rolled" to the desired angle as long as the BBBA was not dry. The embryos were viewed immediately because once dehydrated, the fluorescence faded quickly. For this reason, each sample could only be viewed once before being discarded. The embryos were viewed either with a Leitz Diaplan fluorescence microscope, and records were taken by camera lucida drawing and/or colour photographs (Ektachrome 400 asa) or with a Zeiss confocal microscope, using the Voxel software to process the data.

Bromo deoxy Uridine (BrdU) staining

The method was similar to the method used by Bissen (Bissen & Weisblat, 1989), modified into the following protocol. The teloblast were sometimes injected with lineage tracer at birth as described previously. The development of the embryos was followed, and at the chosen time, the embryos were injected in the macromeres (in order not to disrupt the division of the teloblasts) with 50mM BrdUTP (Sigma), 0.2M KCl and 0.5% Fast Green, and incubated for just over an hour (corresponding to the teloblast cell cycle). During the last 10 minutes of the incubation period, the embryos were exposed to 0.15% pronase E (pronase type XIV, Sigma). This treatment permeabilises the vitelline membrane, supposedly rendering dissection unnecessary.

The embryos were then fixed with 2% formaldehyde in 50mM cacodylate buffer pH 7.3, 1mM $CaCl_2$ for 1h at 4°C, rinsed with cacodylate buffer, and transferred to PBS. In the case of using a peroxidase secondary antibody, the endogenous peroxidase was inactivated with H_2O_2 (3% in PBS). The DNA was denatured with 2N HCl in PBS for 1.5h, and the

embryos rinsed in 0.1M sodium Borate, pH 8.5 to neutralise the acid, then in PBS.

For the anti-BrdU detection the embryos were permeabilised and blocked in PBS/1% TritonX-100/2% BSA (TBP) for 6-8 h at room temperature, then incubated with mouse monoclonal anti-BrdU (Becton Dickinson) diluted in TBP for 16h, and rinsed in TBP for 6-8h with several changes of solution. The secondary antibody was either a peroxidase-conjugated secondary detected with a Fluorescein- conjugated anti-mouse secondary antibody (Cappel: dilution 1/25 to 1/200) or an Avidin-Biotin Complex (ABC) kit. The ABC staining was done as follow.The embryos were equilibrated in PBS for 5 min twice, blocked with 2% Serum Bovine Albumin in PBS for 10 min, then rinsed in PBS. The embryos were then incubated in ABComplex/Horseradish peroxidase (HRP) for 20 to 30 min, rinsed in PBS for 5 min, incubated in 3,3-diaminobenzidine tetrahydrochloride (DAB) for 5 to 15 min, rinsed in double distilled water.

The embryos were counterstained with Hoechst 33258 (5µg/ml) for a few minutes, and mounted in 95% glycerol in PBS.

Cell death staining

The TUNEL method (DNA nick-end labelling of tissue)

I followed the experimental procedure described by Gavrieli (Gavrieli, et al., 1992), with a few modifications as follow, using whole mount or sections.

For the whole mount, the embryos were fixed in fresh 4% paraformaldehyde.

The cells were permeabilised and the nuclei stripped by treating with in 10mM Tris.HCl pH8 for 5 min., followed by an incubation with 20mg/ml proteinase K in 10mM Tris.HCl pH8 for 15 min at room temperature, then washing in water 4 times, for 2 minutes. The endogenous

peroxidase was inactivated with 3% H_2O_2 for 5 min, then rinsed in water. For the DNA elongation, the embryos were preincubated in Terminal Deoxynucleotide Transferase (TDT) buffer 1x (Cobalt-containing buffer from Gibco), then incubated in reaction mixture: 20 µl TDT 5x buffer, 10 µl Bio-21-dUTP 0.5mM (Clonetech 5021-1), 2µl Terminal Deoxynucleotide Transferase (Pharmacia or Gibco, both 16 u/µl) and 68 µl water, for 1h at 37°C. The reaction was terminated by incubation in termination buffer (300 mM NaCl, 30 mM Na Citrate) for 15 min at r.t.

The staining was done using the DAKO ABC system, as in the BrdU protocol. The embryos were counterstained with Hoechst and mounted in 95% Glycerol/PBS.

For sectioned tissue, the embryos were fixed in 4% paraformaldehyde in BT fix buffer (0.15 M $CaCl_2$, 4% sucrose, in 0.1M $NaPO_4$ pH 7.4) in 4°C overnight. The embryos were then embedded in 1.5% agar, 5% sucrose. The blocks were transferred to 30% sucrose, 0.1% azide at 4°C overnight until sunk, and then cut into 15µm cryostat sections, and transferred to 3-Aminopropyltriethoxysilane (TESPA)- coated slides. They were then treated in the same way as the whole mounts, all incubations being performed in humid atmosphere.

Diverse cell death detection methods

Cells dying through apoptosis undergo a well characterised modification of the nucleus and more particularly the chromatin (Wyllie, et al., 1984). In order to detect programmed cell death, a variety of chromatin affinity nuclear stains were used. Different stains had to be tried, and many proved difficult to use because of the high background coming from the yolk: neither acridine orange (10µg/ml), nor propidium iodide (4µg/ml) gave satisfying results.

Daunomycin (5µg/ml, stock solution 100x in kept at -70°C because of its instability), fluoresces in the same channel as rhodamine, therefore

allowing for viewing using confocal microscopy, and showed only negligible background.

Hoechst 33258 (5µg/ml) could be used on live or fixed specimens alike but because it fluoresces in the UV , it was unusable with our confocal microscope. Background was usually low enough, but the staining did not resist the dehydration and clearing used for the lineage tracing detection , and could therefore not be used in conjunction.

The lineage tracer sometimes also allowed the nucleus to be observed very clearly.

Results

Timing of the total period of division of the M teloblast

For further experiments, it was important to know exactly the timing of the division of the teloblasts throughout their blast cell production period. We know that the teloblasts start their cell division cycles as soon as they are born (Bissen & Weisblat, 1989), and there are reports that they divide at the rate of approximately 1h (Wordeman, 1983) to 1h 20min (Zackson, 1982) per cell division during stage 7 in *H.triserialis*. However, there is no estimate of this timing in *H. robusta*, the species used throughout this chapter. On the basis of the timing reported in *H.triserialis*, experiments were done in the M lineage of *H. robusta*, to detect the length of the teloblast cell cycle throughout blast cell production, and the time of the last teloblast division.

The length of cell division at mid-blast cell production was first deduced from the following experiment. In two sets of stage 7 embryos, from which results were pooled, the M teloblast was injected with RDA lineage tracer at age 20h (M=20h) and age 24h (M=24h). After an incubation time, Δt, of 6h (experiment A) or 15h (experiment B), the embryos were fixed, and the number of labelled blast cells was counted in each embryo

(see Figure II.1 A and B). The fact that in both cases the frequency distribution of the number labelled cells is narrow, with the number of labelled cells varying by no more than 3 cells, reflects the fact that the embryos divide at the same rate but are not synchronous. I chose the most frequent number of labelled cells (mode) as reflective of the population's (4 cells in A, 12 cells in B, see Figure II.1 A and B). Because injection is known to induce a lag of approximately 1h for the recovery of the teloblast (Zackson, 1982), I subtracted 1 hour from Δt; i.e. in A, the time between recovery and fixation was 5h, and in B it was 14h. By dividing this time (5 or 14h) by the number of blast cells labelled, I obtained an average time for cell division of about 1h 15min in both cases.

A: 5h/4cells = 1.25h = 1h 15min

B: 14h/12cells = 1.16 h = 1h 10min

This fits Zackson's estimate (Zackson, 1982) of 1h and 18min (at 23°C). Zackson's estimate was obtained in a similar experiment with *H.triserialis* embryos incubated for set periods of time, between 2h and 12h at stage 7. In H.robusta, until M=39h (at least), the M teloblast divides at approximat- ely the same rate. This figure was used for estimating the time required for producing all the cells required in the bandlet for making the 32 segments: 32 x 1h 15min = 40h. After approximately 40h, the teloblast divides to produce supernumerary cells

Based on this estimate, from M=40h on, the M teloblast produces cells that are supernumerary (i.e. cells that later on will not participate in segmentation). But this assumes a constant cycle of cell division. To test whether this was a reasonable assumption, particularly during supernumerary cells production, the same labelling experiment was done in stage 8 embryos, with M teloblast injected with RDA lineage tracer at different times from M=40h onward, and incubated for a Δt of 4h before being fixed and the labelled blast cells counted (see Figure II.2). The Δt was

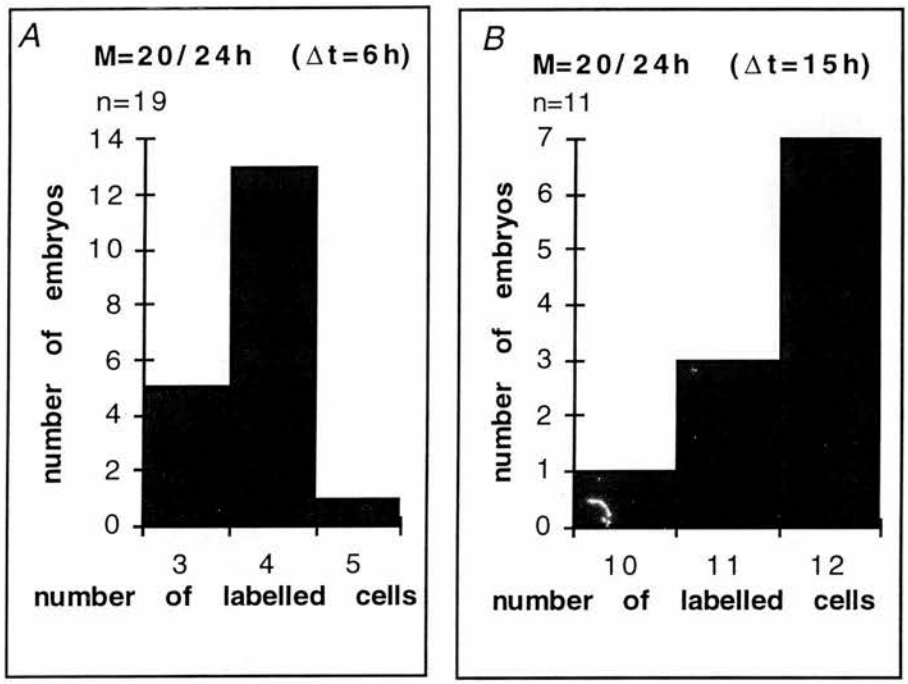


Figure II.1 Histograms of the number of cells labelled after injection at 20 or 24h (data were pooled) and left to develop for 6h (A) or 15h (B). The distribution of the embryos is narrow, reflecting a similar cell division time in the different embryos. In A, the modal number of cells labelled is 4; in B, 12.

kept constant for comparison of the distribution of the number of labelled cells among the different groups.

Up to M=54h (see Fig. II.2 A, B, C and D), the distribution of the embryos is consistent with a cell cycle of 1h 10' or 1h15': there does not seem to be a change in the teloblast cell cycle. From M=55h onward, even though some teloblasts continued dividing at the same rate (7/11), some had stopped dividing (4/11) (see Fig. II.2 E). Before M=55h, a single embryo was found without labelled cells after incubation (Fig II.2 C). It is therefore possible that, in a minority of embryos, the M teloblasts stop dividing earlier.

To confirm the range of time over which the M teloblast stops dividing in the different embryos, M teloblasts were injected at M=52 h (before the majority of M teloblasts have finished dividing). Incubation time was longer, Δt= 12h, until the point at which it was suposed all the teloblasts had finished dividing (see Figure II.3)

Results show a rather broad distribution of the number of labelled blast cells obtained for the embryos labelled at M=52h (see Figure II.3 A). The maximum number of blast cells labelled was 7 and if cell cycle is still approximately 1h15min, this would mean that the teloblast stopped dividing after 8 to 9h. If we take into consideration the 1h post injection lag, it means that the last M finished dividing between at 61h and 62h. The fact that the distribution is now very wide (the number of cells labelled varied between 0 and 7) reflects the fact that the different embryos stopped dividing after a variable number of cell divisions. For comparison, embryos labelled earlier on, at M=20/24h and incubated over 12h differed by 2 labelled blast cells only as opposed to 7 labelled blast cells in the M=52h group (see Figure II.3 A and B).

In order to confirm that no teloblast ever divides after 62h, M teloblasts were injected at M=62h and incubated for 12h . None of the

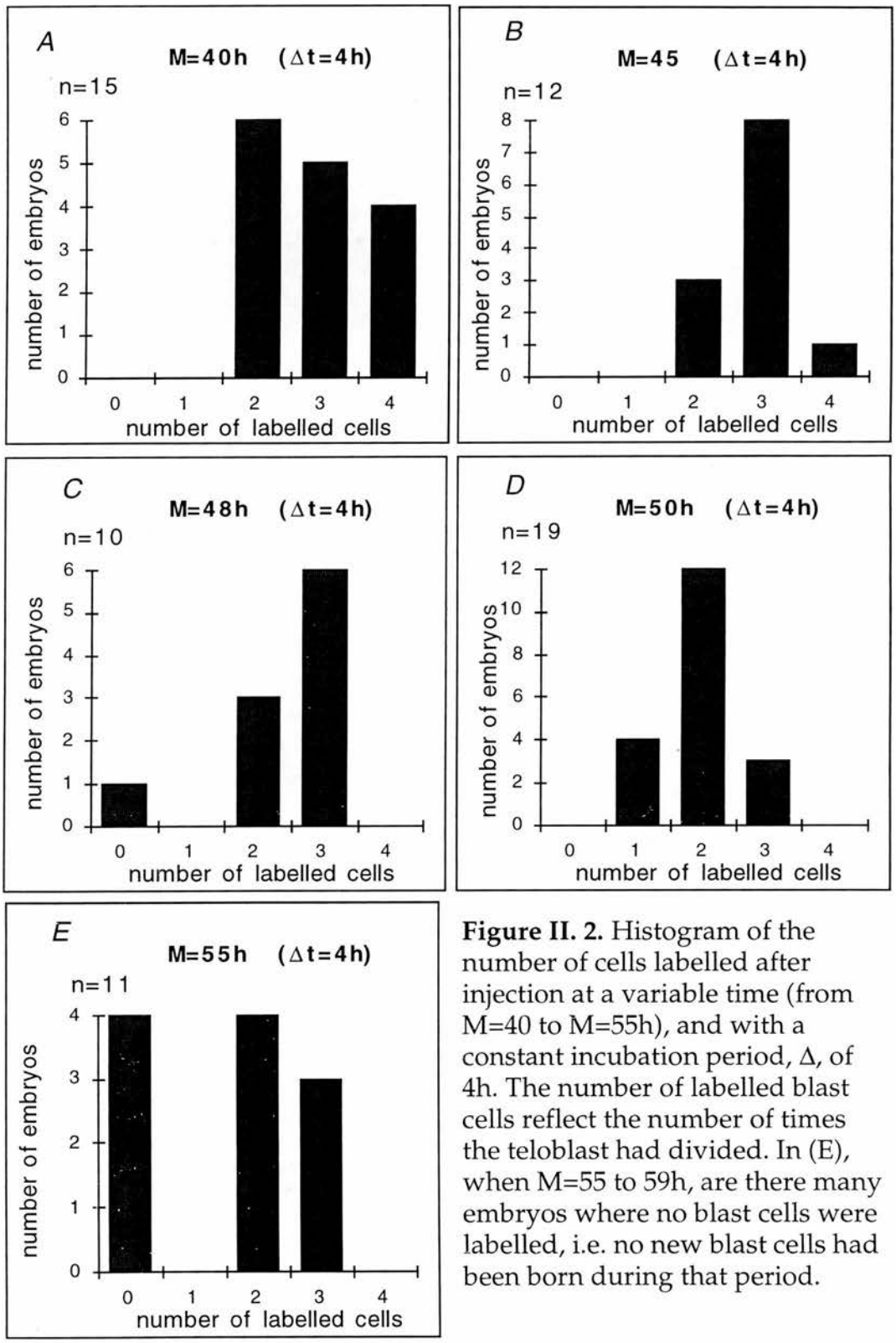


Figure II. 2. Histogram of the number of cells labelled after injection at a variable time (from M=40 to M=55h), and with a constant incubation period, Δ, of 4h. The number of labelled blast cells reflect the number of times the teloblast had divided. In (E), when M=55 to 59h, are there many embryos where no blast cells were labelled, i.e. no new blast cells had been born during that period.

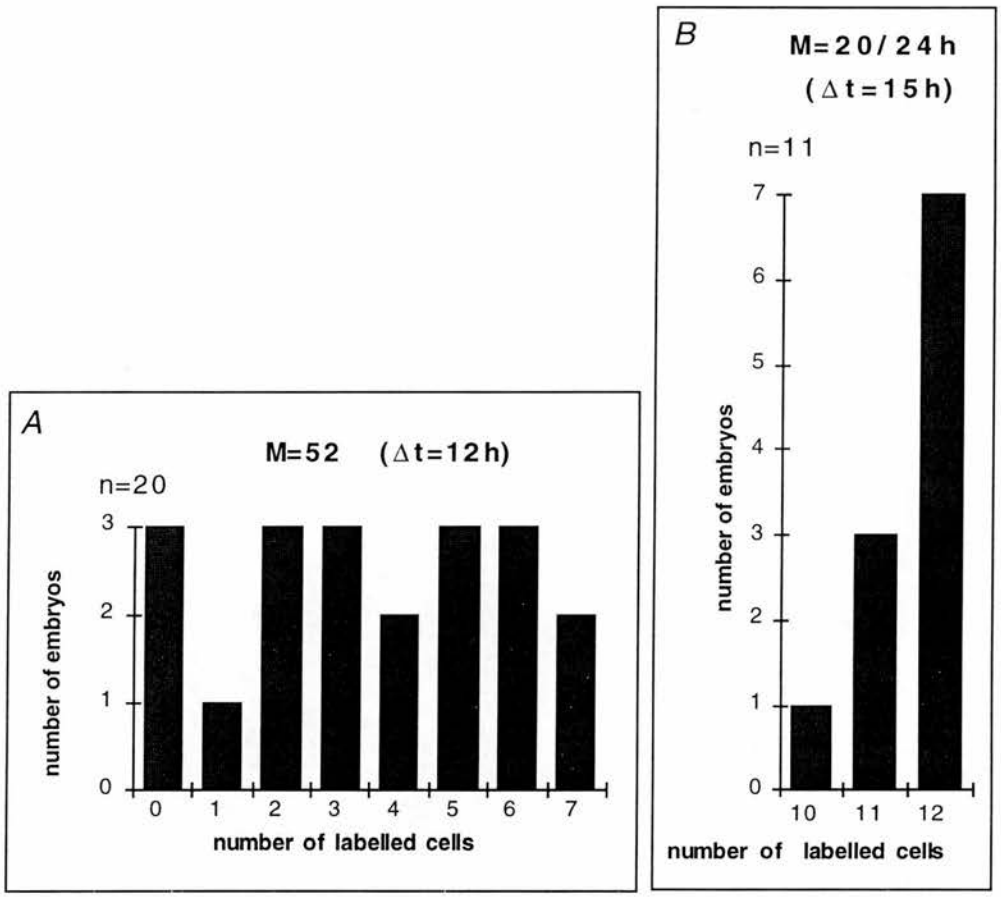


Figure II.3. (A) Histogram of the number of cells labelled after injecting the M teloblast when M=52h, and incubating the embryos for 12h (until M=64h). (B) Figure II.1(B) is reproduced here for comparison. The distribution of the embryos in (A) is much wider than in (B) even though their incubation time, Δt, is very close.

embryos showed any labelled blast cell. Among the 20 animals observed, at least 11 showed a healthy labelled M teloblast (indicating that the lack of labelled cells was not due to damaging injection or no injection). Therefore, M teloblast division has stopped at M=62h at the latest.

Injection into the teloblast has the disadvantage that it can disrupt the cell cycle. The teloblast can take 1h to recover from the injection. The two consequences are that (i) the imprecision detected in the timing of the last teloblast might come from the teloblast itself (i.e. teloblasts from different individuals might stop dividing at a variable time), or from the experiment, some teloblasts being more affected by the trauma of the injection than others, and taking more or less time to recover before dividing, and (ii) the traumatic effect of injection might increase with the age of the teloblast, and late injections might disrupt the cell cycle and this might be the cause to teloblasts stopping dividing.

I attempted to use BrdU as an alternative, non-invasive method for detecting the last teloblast division. BrdU is a small molecule, known to diffuse between the cells of the leech embryo (Bissen & Weisblat, 1989). The injections required for this experiment (into the teloblast at birth and into the macromere, see Material and Methods) are not as disruptive as the injections performed in the previous experiments, since they are done either much earlier than the time of detection of the teloblast cell cycle (48h to 70h earlier), by which time the teloblast has recovered, or in an adjoining cell to the studied teloblast (macromere). In my hands however, BrdU was never detected.

Variation of the number of blast cells in the bandlet during the setting up of segmentation

The blast cells produced during the division of the teloblasts first form a column, or bandlet, that progresses anteriorly as more cells are added to it posteriorly. During early stage 7, all the bandlets from each side

join together in the germinal band, and they progress together anteriorly, up to a point where at early stage 8, the left germinal band and the right germinal band join to form the germinal plate (Fernández & Stent, 1980). The blast cells converge to these two points, entering these morphological landmarks at a bend (see Figure II.4).

The number of blast cells from each teloblast in the germinal band and germinal plate increases steadily from 0 to 32 (or 64). I assumed that eventually the number of primary blast cells from one teloblast in the germinal band and germinal plate is 32. This comes from the observation that one (or 2) blast cell is (are) at the origin of a clone making one hemisegmental complement (Zackson, 1982): each mesoblast cluster is a clone derived from a single primary mesoblast, as well as being a precursor to an adult hemisegment (Weisblat and Shankland, 1985) ; in the M, O and P cell lines, one blast cell generates one segmental complement of progeny; in the N and Q cell lines, 2 blast cells are required to generate one segmental complement of progeny, and the leech embryo comprises exactly 32 segments. In contrast to the germinal band and germinal plate, in the single bandlet the cells are constantly entering (as the teloblast divides) and leaving (as they enter the germinal band) (Figure II.5).

Because the supernumerary blast cells are cut off at the point of junction between single bandlets and germinal band (Shankland, 1984), I undertook to count the number of blast cells present in the bandlet posterior to the germinal band. The purpose of the observation was to find out if any change occured in that number and whether that number was constant over time and between embryos. I observed this dynamic structure by lineage tracing: one teloblast or two contralateral teloblasts were injected with RDA, and the embryo left to develop for a time sufficient for most of the blast cells that will be part of the segmental body to be born and enter the germinal band. The embryos were then fixed and the single bandlet structure

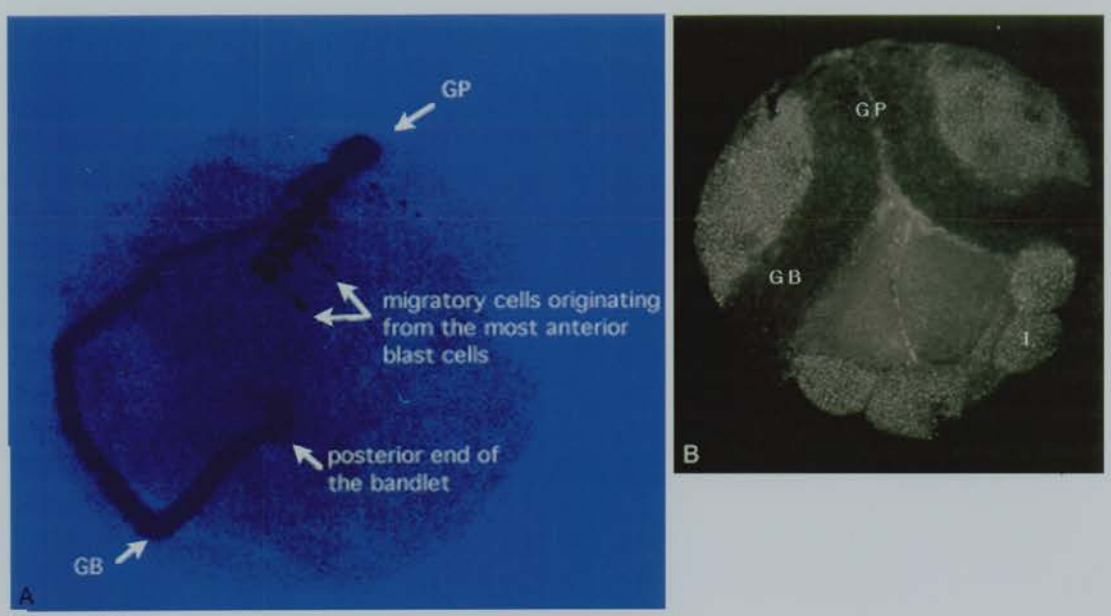


Figure II.4

Figure II.4 The blast cells become part of 3 morphologically distinct structures: the bandlet, the germinal band (**GB**) and the germinal plate (**GP**).(A) One lineage through a clarified embryo, stage 8, dorsal view. The embryo was injected with lineage tracer in the N teloblast, and left to develop until enough blast cells were produced to invade the bandlet, the germinal band and the germinal plate.(B) A ventral view of a stage 8 embryo. The teloblasts (T) can still be seen at the posterior end while the germinal bands are zipping up.

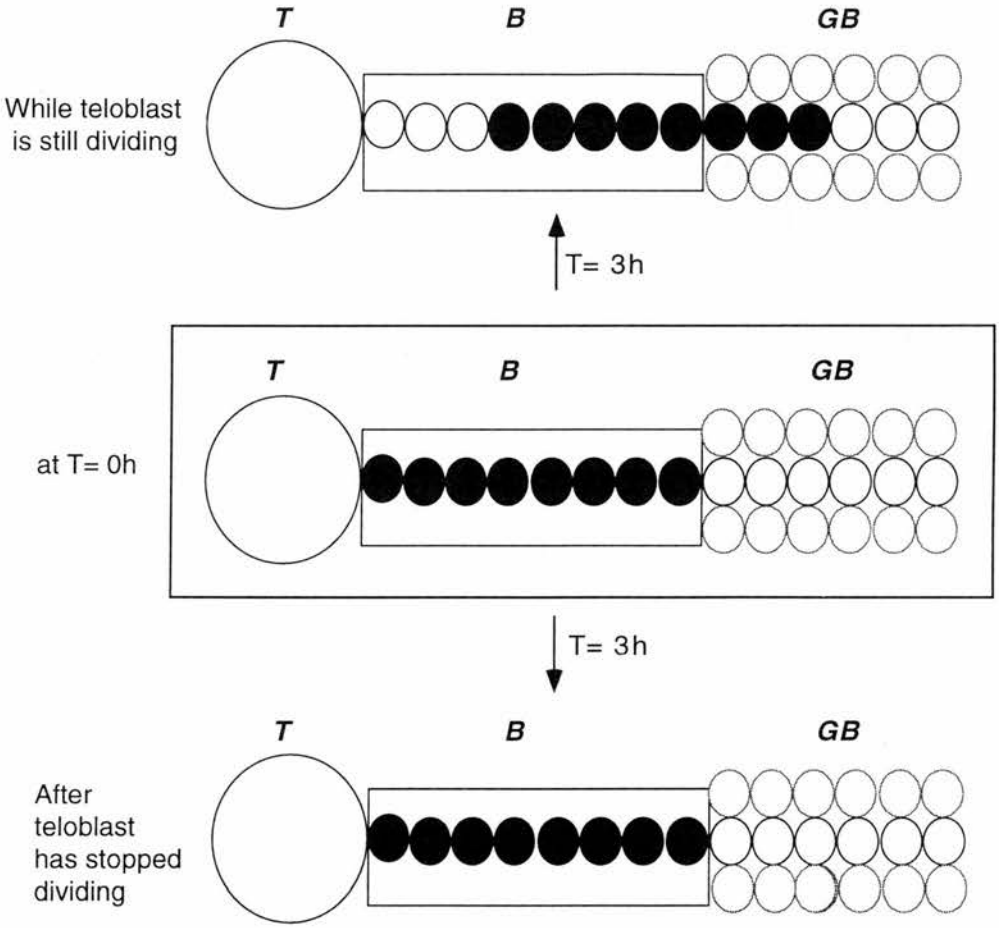


Figure II.5 While the teloblast is still dividing, the bandlet is a dynamic structure, gaining cells at the posterior end where the teloblast divides and lo sing cells at the anterior end, where the cells enter the germinal band. After 4 hours, the germinal band has acquired 3 more cells (the teloblast divides at a rate of 1 per hour). My results show that on average, it will also have lost 4 cells to the germinal band, considering that the net size of the bandlet neither increases nor decreases. Until detachment, long after the time the teloblast has finished dividing, the number of cells in the bandlet was not seen to vary significantly. However, this number was variable from on embryo to another and even from one contralateral side to the other.

T: teloblast, B: bandlet, GB: germinal band

observed. The number of blast cells present between the teloblast and the germinal band was counted. The germinal band could be highlighted in two ways: either by counterstaining for nuclei with Hoechst, thus making the other bandlets appear as well, and the point of convergence into the germinal band could be determined that way, or by observing the angle made by the RDA-labelled bandlet as it enters the germinal band. With both methods, there was some imprecision about determining the point where the bandlets were joining. With the first method, I observed that the bandlets converge slowly (see Figure II. 6, D and E.). Because the stain was only within the nuclei however, I could not observe cell contact. As a result of fixation, it is also possible that the structure was not so well preserved, thus detaching the bandlets from each other. I estimated the error made at this point to be between 0 (when the point of convergence was clear, usually in late stages) and 3 cells (when the point was not so clear). There is unfortunately no molecular marker known for the germinal band, distinguishing it from the more posterior single bandlets. In the second method, consisting of detecting the angle of entry, the same imprecision was met, because (especially in young embryos), the angle was not acute, but aften like a curve, and contained many cells (see Figure II.6, A, B, G). In the same way, I had to decide where the bandlet was ending (corresponding to where the germinal band was starting). Finally, not all the embryos were as easy to quantify, due to the differences in the quality of the labelling. The differences might have been brought about by dye batch differences, quantity of dye injected, time between fixation and observation. I estimated the error to be about the same as previously, between 0 and 3 cells, depending on the angle.

In total, between 100 and 140 embryos were injected for each of the M, the N and the OP lineages, and only 20 for the Q lineage. Among those, some were fixed too early (no supernumeraries yet), or too late (after

Figure II.6 The point of entry of the bandlet into the germinal band is not always detectable precisely. Embryos were either injected with lineage tracer and scored for sharp angle of entry into the germinal band, or labelled with a nuclear marker and scored for convergence of the nuclei from different lineages. Arrowheads represent site of entry or range of site of entry of the bandlet into the germinal band. (A, B, C) Stage 8 embryos, in which teloblast N was injected with lineage tracer at birth, and fixed 76h later. Note how the angle is different in each sample. (D,E) UV illumination of a Hoescht staining of stage 8 embryos in which proteloblast OP was injected at birth and fixed 49h (D) or 44h (E) later. Note how the O and the P lineage are very close to each other from the time of birth, even though they seem to converge with the other lineages only later on. (F, G) Stage early 8 embryos in which the M teloblast was injected at birth and incubated for 36h (F) or 40 h (G) before being fixed. Note how in one of the samples the angle of entry is well defined and how it is less well defined in another. T: teloblast, B: bandlet, GB: germinal band.

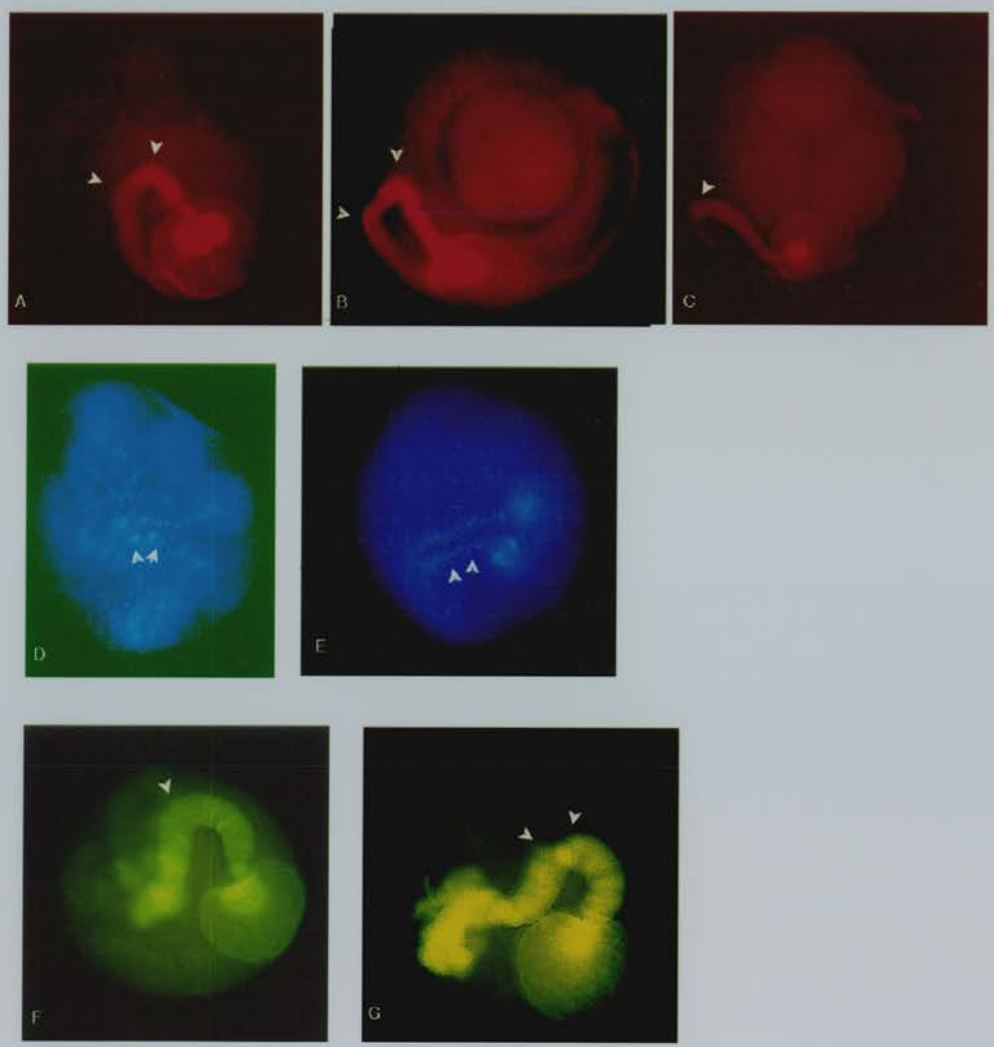
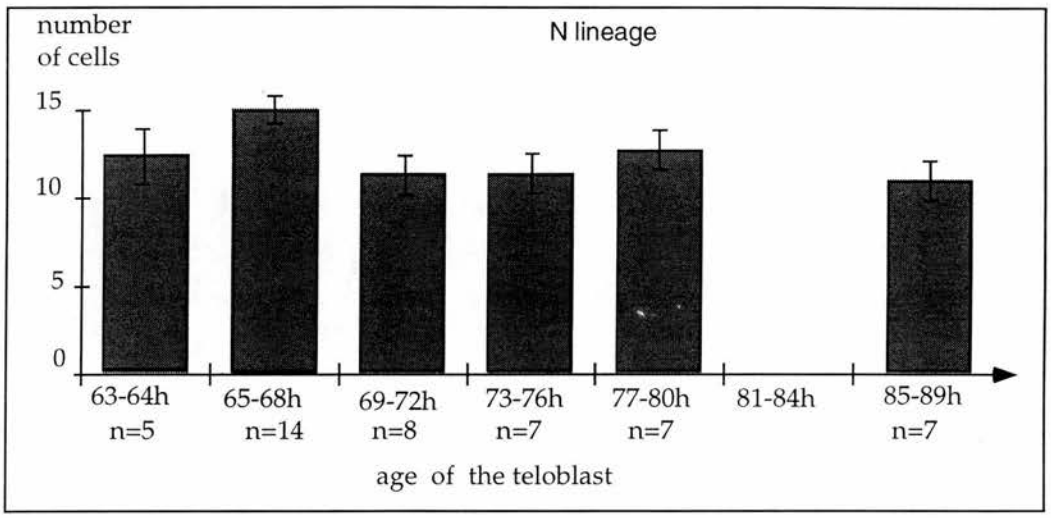


Figure II.6

detachment of the bandlet) to be used. Among those, only a small proportion could be used for counting the blast cells, when the quality of the staining was good enough for the imprecision to be minimum. Some of the embryos were injected on both the right and the left side, and data were compiled from both sides because they were usually found to be different (in bandlet morphology as well as in number of blast cells).

After injection at the time of birth of the teloblast (t=0), the embryos were left to develop at 23°C in order to let the teloblast divide at the known rate. Each clutch contained most often around 20 embryos and they were fixed in batches of 6 at different times, differing by at least one hour i.e. one blast cell birth. The time chosen for fixation was between the theoretical time of last segmental blast cell birth and the time of detachment of the bandlet from the germinal band. Information from all the different clutches were pooled for each "age" (i.e. time since birth of the teloblast, see Material and Methods), and compared. Only the M lineage (1 blast cell per hemisegmental complement; M is the earliest born teloblast) and the N lineage (2 blast cells per hemisegmental complement; N teloblast is born 4h after the M teloblast and 10h before the last teloblast) were studied in such detail (Results are presented in Figure II.7). For the other lineages, I only determined the time of detachment.

When I compared in lineage M and N the number of blast cells at different ages (using a parametric Anova: analysis of variance), I found no significant difference between the different ages for M ($p=0.2504$). For N, I found that there was a very significant difference among some of them ($p= 0.0018$). I used a Tukey-Kramer comparison test to find out which group was the most different. The group 65-68 was considered to be significantly different from the groups 69-72, 73-76, and 85-89. Even though the length of the bandlet was found to vary (with significantly more cells in the bandlet between N=65h and N=68h), no progression was observed in the



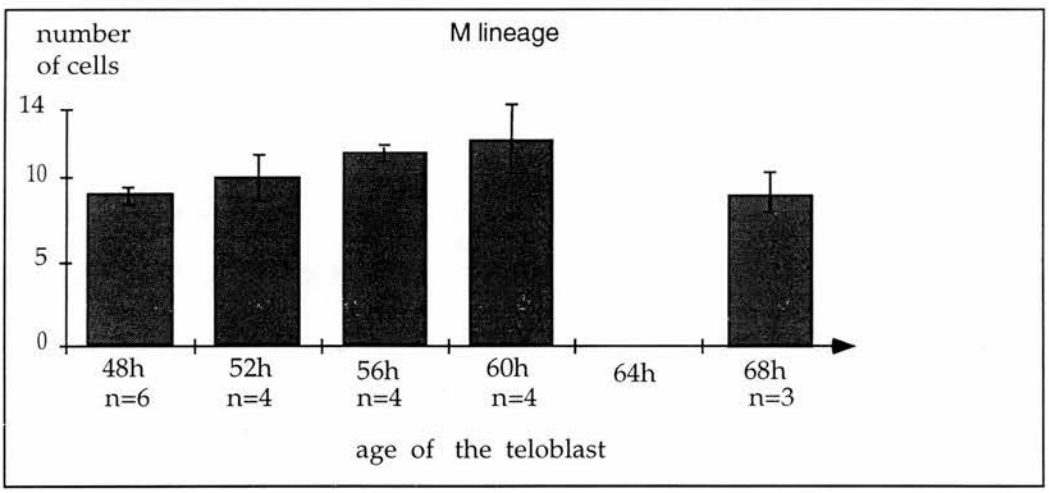


Figure II.7 Variation of the number of blast cells in the single bandlet. Histogram of the number of cells in the single bandlet of the N and M lineages, during the late stages of teloblast division but before the bandlet detach. In the N lineages, the observations were made about every 4 hours and grouped around an average time. The times corresponds to the age of the teloblast. which correspond to the number of hours since it started producing blast cells. No observations were made in the group 81-84h in the N bandlet and in the group 64h in the M bandlet. n is the sample size in each group of observations. The error bars are the standard errors.

lengthening or shortening of the bandlet over the period N= 63h to N=89h. Where could this variation come from? The M teloblast divides regularly until it stops. If the N teloblast does the same, then at this stage (the N teloblast is still producing segmental blast cells), the lengthening of the bandlet would be due to a retard in integration rather than a speeding up of the cycle of the teloblast. It is possible that between 65 and 68h old, the N lineage changed its orientation in such a way that the number of blast cells in the bandlet was always overestimated, or it is possible that the bandlet slows down its integration into the germinal band (around 65h), and then "catches up". The variation might also be due to the fact that the variation observed is a result of both the actual variation of the number of cells in the bandlet and the error in counting these cells.

These results show that the number of cells in the bandlet may vary significantly during the last cell divisions of the teloblast in the N lineage but not in the M lineage. At the time of detachment, the number varied from 8 to 10 in the small sample of M lineages studied and between 7 and 15 in the small sample of N lineages studied.

Timing and observation of the supernumerary blast cells produced by the M teloblast

Another experiment was done to assess the number of supernumerary blast cells actually produced, only in the M lineage. For this I double labelled a single bandlet, once at the time of birth of the teloblast and once at t=32h. I then left the embryos to develop for Δt=16h, 20h, 24h, 28h. The drawings of the resulting labelled embryos are presented in Figure II. 8

Consistent with the results presented in the first paragraph (see Fig. II. 1, 2 and 3), all the blast cells were never produced at M=32h, as some double labelled cells were always detected in the germinal band (see Figure II. 9). It was therefore not possible to calculate simply the total number of

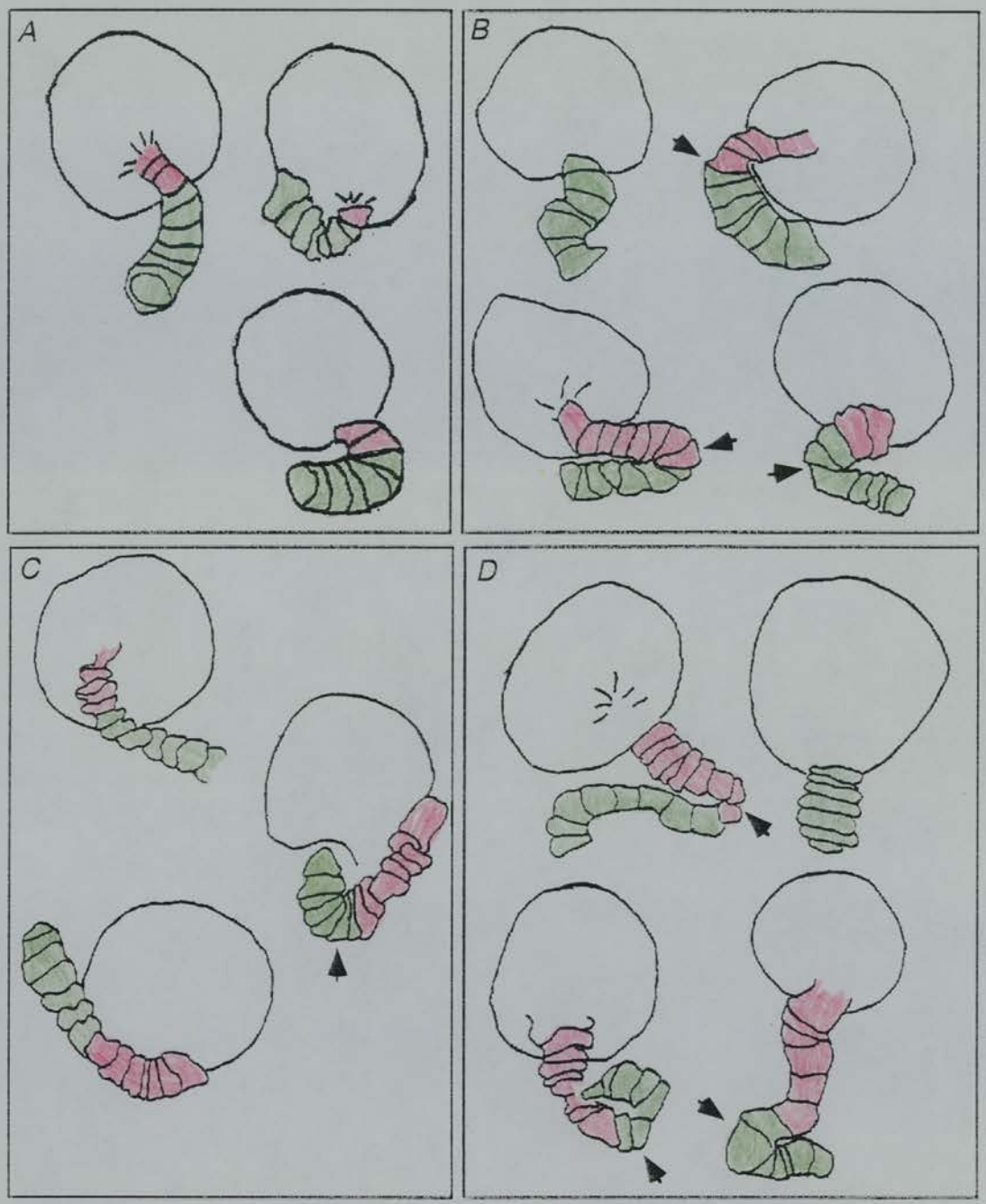


Figure II.8: Camera Lucida drawings of M teloblasts and their progeny, injected with lineage tracer at M=32 and incubated for different Δt. (A) Δt= 16h (labelling of cells born between M=32h and M=48h), the 3 teloblasts produced 8, 9 and 10 labelled blast cells; (B) Δt= 20h (labelling of cells born between M=32h and M=52h), the 4 teloblasts produced 7, 9, 11 and 15 cells; (C) Δt= 24h pulse (labelling of cells born between M=32h and M=56h), the 3 animals show 11, 14 and 15 cells; (D) 28h pulse (labelling the cells born between M=32h and M=60h) the 4 teloblasts produced 7, 12, 17 and 14 cells. Arrows point to the "bends" where the cut off between supernumerary and segmental cells might happen.
In green are represented the putative segmental cells, and in red the putative supernumerary cells.

cells produced by the teloblast by counting the labelled cells as being supernumerary (i.e. coming after the 32nd -segmental- blast cell). However, at M=32h, the teloblasts are still synchronous (see above), and divide at the rate of approximately 1h15' per cell division. Therefore the number of blast cells already produced by the teloblast at the time of the second injection, must be on average 25 cells.

In all of the embryos in the different group (3 to 4 embryo per group), all produced at least 7 blast cells during the incubation period (Figure II.8), the minimum number required for making the 32 segment founder cells.

In embryos from M=32 to 52h and in M=32 to 60h, some embryos differ by more than 2 labelled cells, as would be expected if all the teloblasts were still dividing at the time of fixation (see Figure II.1): Figure II.8 shows the number of cells produced by the M teloblast between t=32h and a variable time. After 16h, the number of new blast cells varied between 8 and 10 cells (n=4); after 20h, the number of new blast cells varied between 7 and 15 (n=4); after 24h, the number of new blast cells varied between 11 and 15h (n=3); after 28h, the number varied between 7 and 17 cells (n=4). Therefore, some specimen must have finished dividing before others. This was expected from the results presented above, although some embryos seemed to have finished dividing earlier than the 52h limit suggested in the first paragraph. However, the number of embryos on which this is based might not be as representative as the number on which the previous result is based.

The overall number of double labelled blast cells is always smaller than the number of blast cells expected for the time of incubation (Figure II.8B). It is therefore possible that (i) the embryos had always all stopped dividing at the time of fixation or (ii) there is a temporary stopping at 40h, in the transition between making the segmental and the supernumerary blast cells.

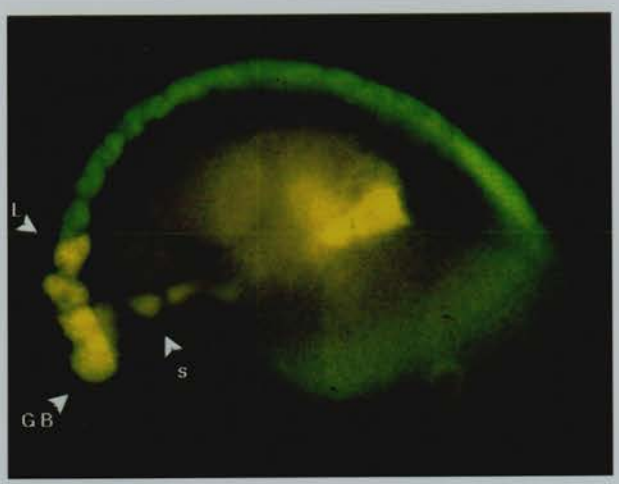


Figure II.9

Figure II.9 Stage 8 embryo (73h after birth of the M teloblast), double labelled. The teloblast was first injected after its birth with FDA, then 32h later with RDA. The most anterior blast cells, born before 32h are only labelled by the FDA (green), whereas the cells born after 32h are double labelled (yellow). Some of the double labelled cells are in the germinal band and have undergone division, forming metameres in the same way as the more anterior ones. At this stage (73h after birth of the M teloblast), the supernumerary cells are distinct from the segmental ones, and the bandlet can be seen detaching. L: limit of double labelling, GB: start of the germinal band, s: supernumerary cells

There is no obvious mophological distinction between the segmental blast cells and the supernumerary blast cells at the times observed (i.e. between M= 48h and M=60h), neither is there any morphological change in the supernumerary blast cells during this period. However, when there is a bend in the bandlet (7 embryos/14), this corresponds to the segmental/supernumerary point of separation (see Figure II. 8A). The "bend" at the junction does not form in all the embryos at the same time. Although in some of the embryos the number of supernumerary blast cells seems sufficient to push the segmental cells down into the germinal band, in some with a smaller number of blast cells (e.g. none or 2 supernumerary cells, see Figure II.8. B or D), this cannot happen.

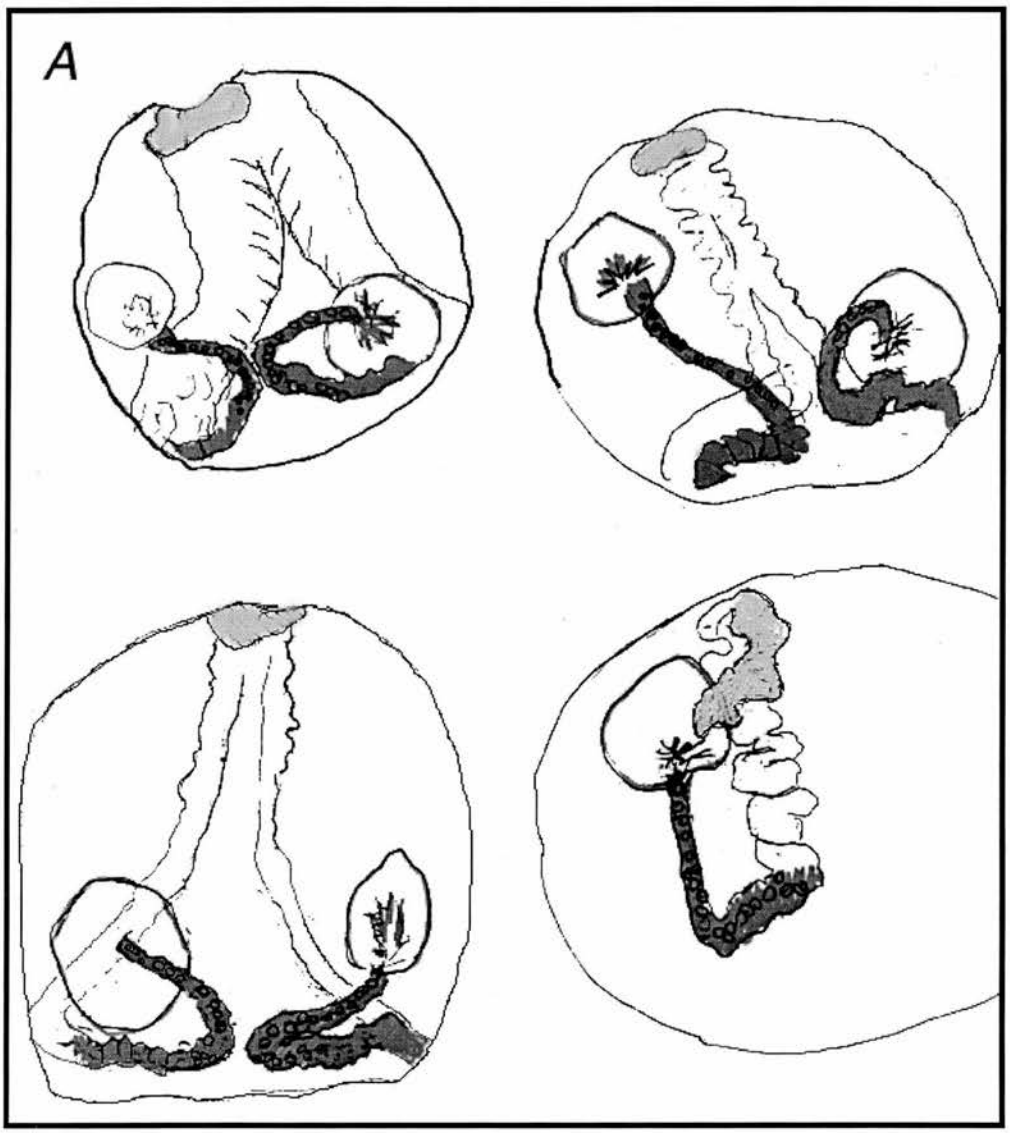
It is therefore difficult to assign a role to these supernumerary cells considering that sometimes they are present, and sometimes they are not, and when they are, the shape that the bandlet takes is variable.

Timing of separation of the supernumerary bandlets from the germinal band

In order to observe the detachment, I used lineage tracing in the same manner as previously, and used the same experimental embryos. The embryos were fixed at different times and whether the bandlets were detached or getting detached was noted.

I found that the embryos were consistent in their timing of detachment between batches. For example, see Figure II.10: the N teloblast was injected and its progeny (the N bandlet) was followed in time by fixing the embryos after different Δt as previously. At t=72h, the point of entry of the bandlets into the germinal band is recognisable by the angle of the bandlets, but the angle is not the same in all the embryos. At t=75h and t=80h, the angle becomes more accentuated as the germinal band are zipping up more anteriorly. In the embryos fixed at 100h, 3 out of 4 embryos show detached bandlets (on each side) and still show cellular debris, while the 4th embryo is clearly ready to detach.

Figure II.10 Camera lucida drawing of labelled N lineage embryos fixed at different times of development. Embryos were injected with lineage tracer just after the birth of the N teloblast so that all its descendents should be labelled.The most posterior part of the embryo, i.e. the part containing the single bandlets and the teloblasts, is represented in dark grey, whereas the more anterior labelled cells, wrapping around on the other side, are in light gray. Black dots represent the nuclei of the blast cells. All embryos are late stage 8 to early stage 9. (**A**) embryo fixed 72h after the birth of the N teloblast (**B**) embryo fixed 75h after the birth of the N teloblast, (**c**) embryo fixed 80h after the birth of the N teloblast (**D**) embryo fixed 100h after the birth of the N teloblast. Anterior is at the top.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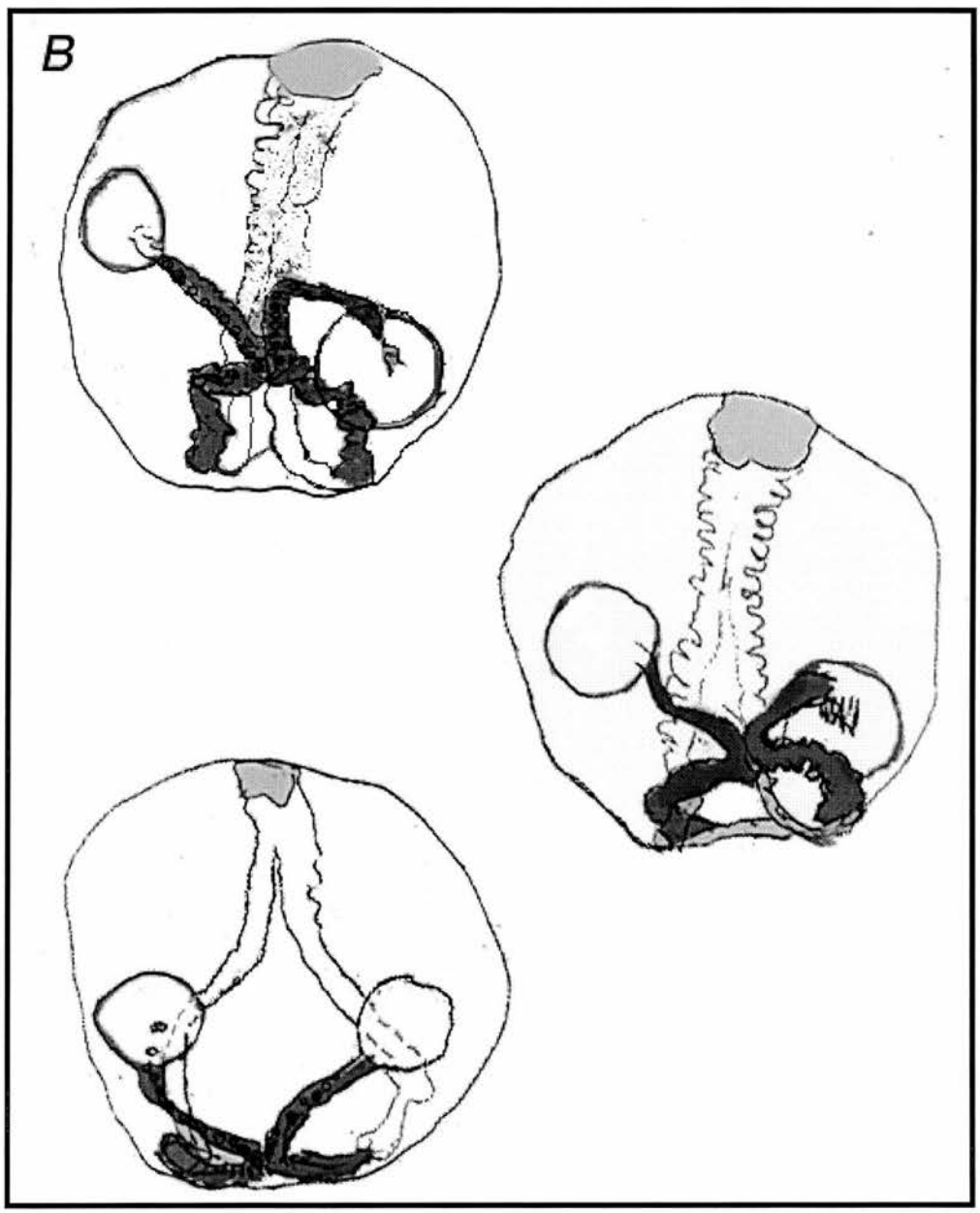
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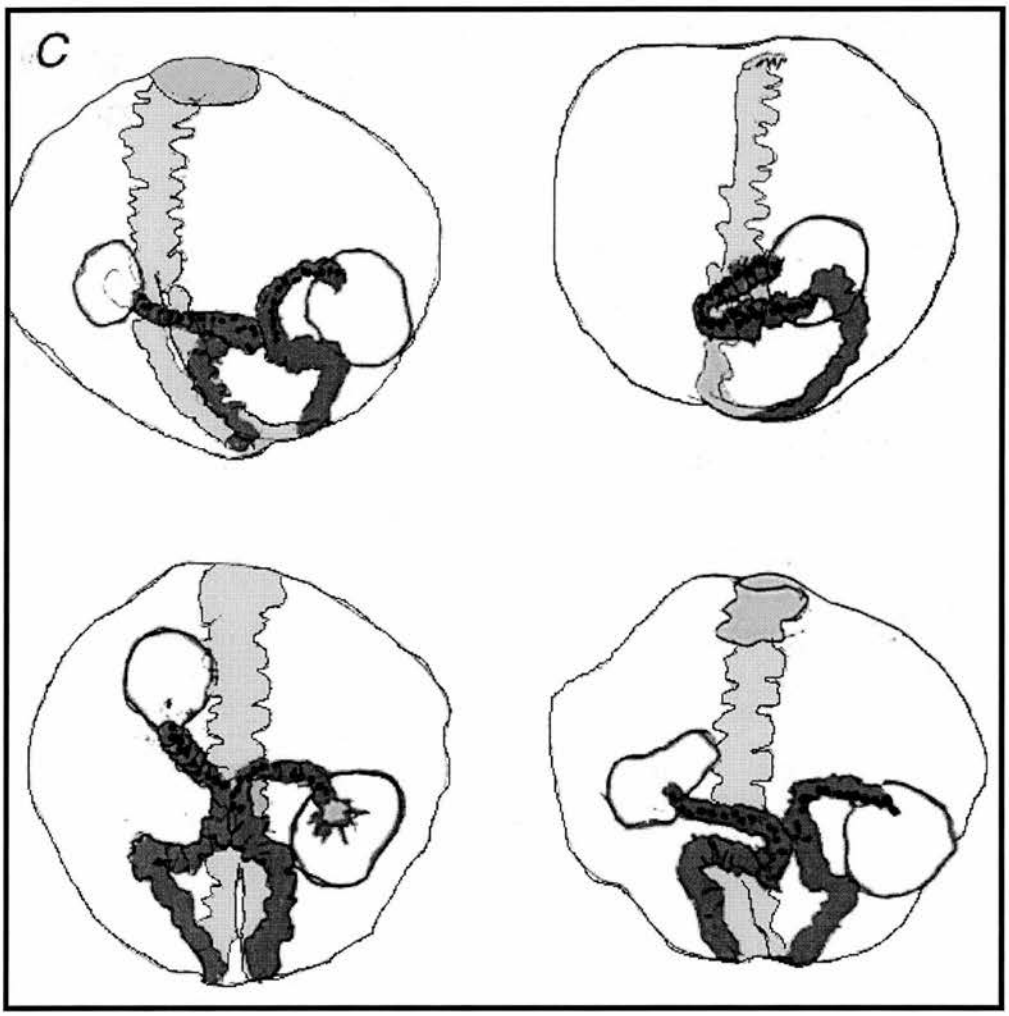
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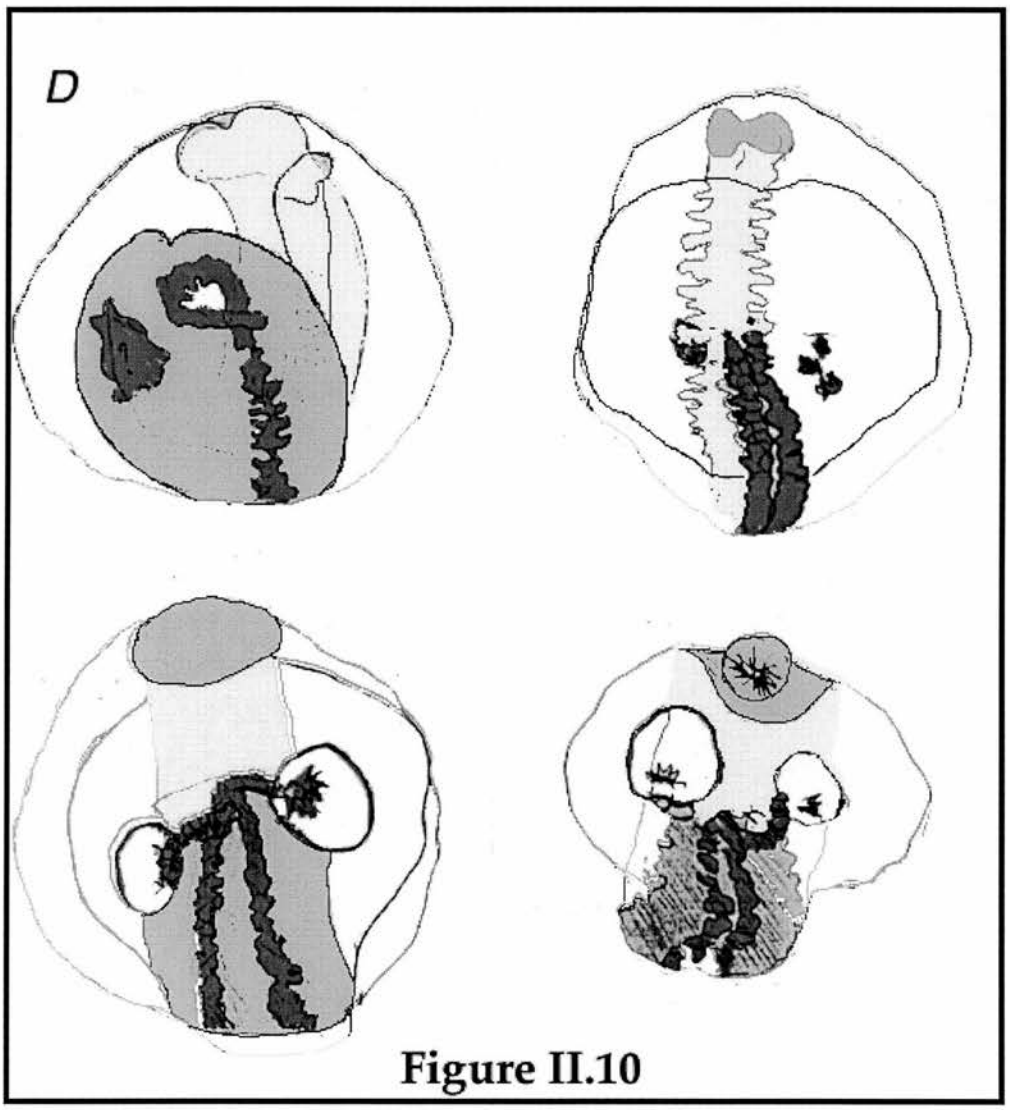

Figure II.10

It is interesting to note that from N=75 onwards, the left and the right bandlets are in contact posteriorly, even though the more anterior left and right bandlets haven't joined yet into the germinal plate. It is not possible, on the view of these sole drawings, to determine (i) if they are in contact via gap junctions, and (ii) if it is the same cells that stay in contact or if the zone of contact moves as the germinal plate progresses posteriorly.

The detachment in the M lineage was around 70h (detachment was observed in embryos between 67h and 72h) after the birth of the teloblast. In the O and the P lineages detachment occurs between 66h and 73h after the birth of the O/P proteloblast. The Q was never followed late enough to determine the exact time of detachment (the bandlet hadn't detached at 92h, the latest observation), but the timing is probably closer to the N timing, considering it has to produce 64 segmental primary blast cells too (see the graph, Figure II.11).

In the process of detaching, some cells observed at the site of detachment show an altered morphology (see Figure II.9): 2 or 3 cells at the joining between the germinal band and the single bandlet are elongated. These cells seem to die quickly and disappear whereas the more posterior cells, still organised in a bandlet, stay for longer. The dying cells were not detected in all the embryos were the bandlets were detaching (1/8 in the N lineage around 100h, time of detachment; 4/16 in the M lineage between 67 and 72h), and the death of these cells can therefore be interpreted as a relatively fast process.

Double labelling experiments, such as injection of the M teloblast and the OP proteloblast or M and N teloblasts showed that at the time of detachment, the lineage detaching is aligned with the previously detached lineage, i.e. M is not in register with any lineage when it detaches; O and P are in register with each other and with M when they detach; N is in register with M, O and P when it detaches. This means that the bandlet always

Figure II.11 Chart of the timing of the bandlets
I used known data about *H.triserialis*, a species very close to *H.robusta*, in which the cell cycles of the early embryo have been worked out (Bissen and Weisblat, 1989, Dev. 105, 105-118), except in the M teloblast where the cell cycle was established at 1h15' experimentally. On the basis of the teloblasts cell cycle timing and the number of segmental blast cells produced, I worked out a theoretical time at which to expect the supernumerary blast cells to be born. Landmarks were calculated to see if any correlation could be found between known events and behaviour of blast cells in vivo. I have added information collected about the timing of separation of the supernumerary bandlets from the teloblasts.

Figure II.11 Chart of the timing of the bandlets
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* indicates the observed time of detachment of the bandlet from the studied lineage
Δ indicates the appoximate time of last teloblast division

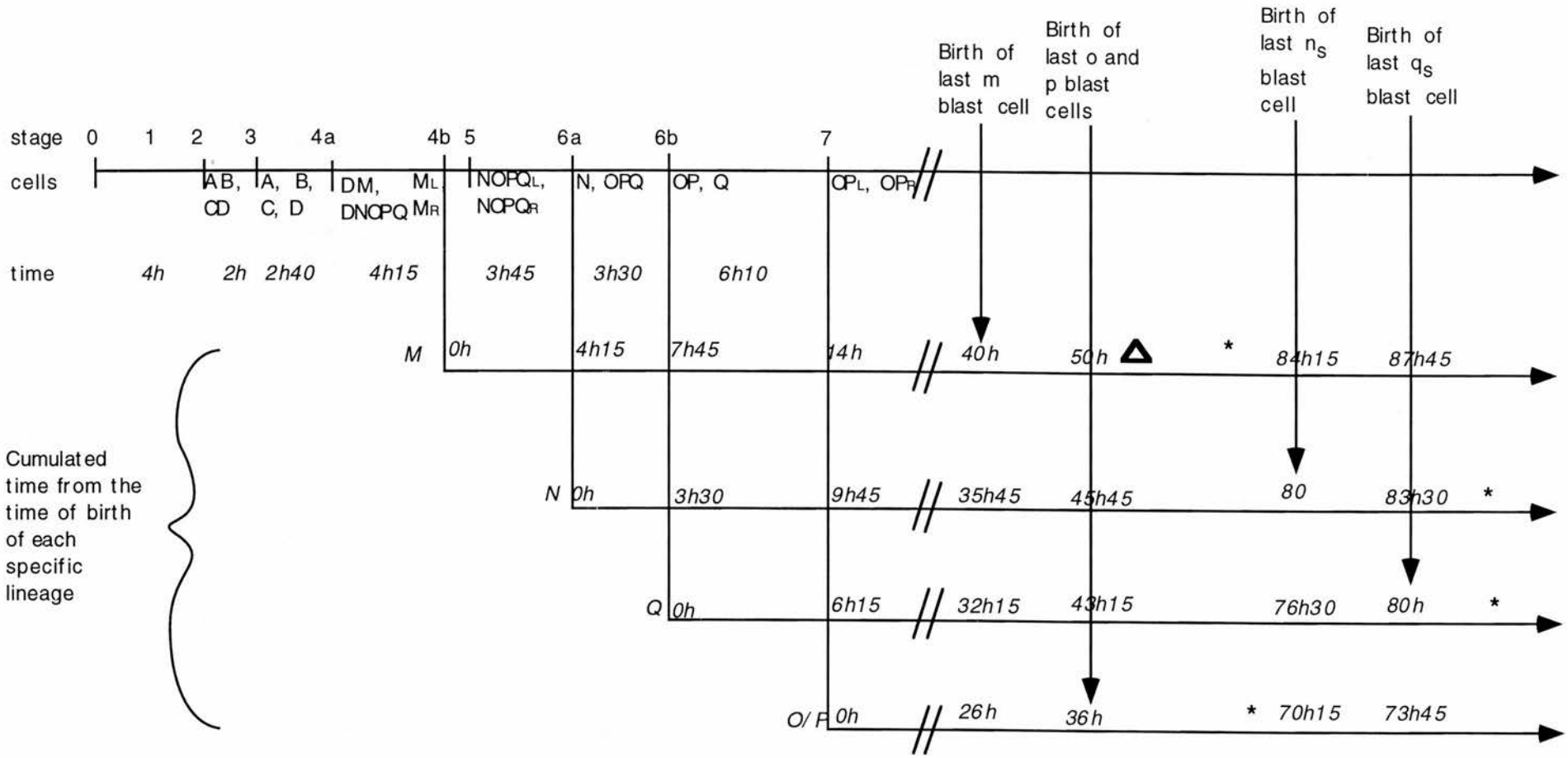
stage 0 1 2 3 4a 4b 5 6a 6b 7
cells
AB, CD
A, B, C, D
DM, DNOPQ
ML, MR
NOPQL, NOPQR
N, OPQ
OP, Q
OPL, OPR
time
4h
2h
2h40
4h15
3h45
3h30
6h10
Birth of last m blast cell
Birth of last o and p blast cells
Birth of last ns blast cell
Birth of last qs blast cell
M 0h 4h15 7h45 14h 40h 50h 84h15 87h45
N 0h 3h30 9h45 35h45 45h45 80 83h30
Q 0h 6h15 32h15 43h15 76h30 80h
O/P 0h 26h 36h 70h15 73h45
Cumulated time from the time of birth of each specific lineage

Figure II.11

detaches at the border between germinal band and single bandlet; the bandlets are in register. The order in which the bandlets detach corresponds approximately to the order in which they are born and the order in which they have finished producing their blast cells (see Fig. II. 11): M, then O and P, and finally N and Q. At the time the bandlet detaches, it is aligned with the older lineages.

Supernumerary cells follow a distinct differentiation path than the segmental cells

I observed the cells in the M bandlets at different times, trying to find any distinctions between the supernumerary blast cells and the segmental blast cells before their position would give away their fate. From the previous experiments, it is known that the M teloblast takes 1h 15min per cell division, and that all M teloblasts have stopped dividing at 60h. It is therefore possible from these figures to know at any time whether the cells in the single bandlets are destined to become segmental or supernumerary. There is a lag between the teloblast's last cell division (60h latest) and the time of detachment (around 70h)

Supernumerary blast cells rarely divide

In the M lineage, the teloblast has finished dividing between 48 and 60h, but the bandlet doesn't detach before 70h. The first supernumerary blast cell is produced around M=42h. This means that the supernumerary blast cells would have time to divide (18h maximum for the most anterior one), considering that the cell cycle of the primary blast cells is 8h (Bissen & Weisblat, 1989), and the subsequent (secondary) blast cell 5h (Bissen & Weisblat, 1989). I therefore looked for mitotic figures and signs of cell division in the single bandlet around the time the supernumerary blast cells would be expected to divide if they were following the same cell cycle as the segmental blast cells (see Figure II.12). Only very rarely (2/17 in the 64h until 72h group of observations) did I observe divided cells in the single

Figure II.12 The supernumerary cells do not divide. Confocal image of a 70h old RDA labelled M bandlet. The embryo is at stage 8. Anterior of the bandlet is to the top. The teloblast is not visible but was just posterior to the most posterior visible cell. In this batch of injections, the nuclei were heavily stained with RDA (red and yellow dots).7 or 8 nuclei are visible at the posterior end, arranged more or less in a line. They all come from primary blast cells that haven't divided, as opposed to the more anterior nuclei (lighter pink) which are grouped, and belong to secondary or terriary blast cells. Objective x40, zoom x 20, scalebar approximately 20µm. Collection of 29 sections, over a total of 40µm.

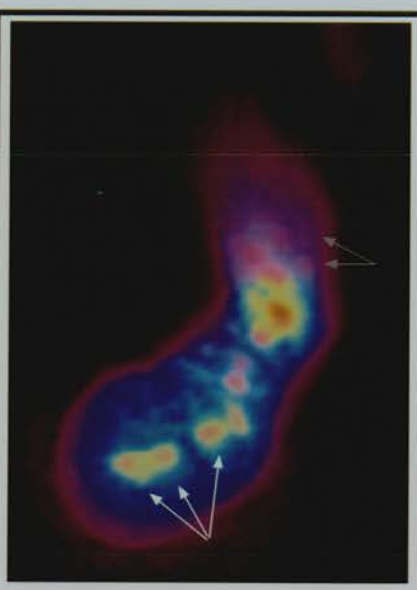

Figure II.12

bandlet, and this was never more posterior than the 3rd cell after the germinal band. The cells attached to the teloblast as they separate from the germinal band are never divided (see Figure II.9).

In the N lineage, the supernumerary cells have a maximum of 30h to divide, and in the same way, no cell division is observed even though the cell cycle of the primary N blast cell is 22h for nf and 28h for ns (Bissen & Weisblat, 1989).

In the O and the P lineages, the supernumeraries have a maximum of 30h to divide, and no cell division is observed in the bandlet, yet the cell cycle of the primary blast cells is 21h.

Supernumerary blast cells do not exhibit characteristic apoptosis

At a different time in the different lineages, the bandlets detach from the germinal band. What happens to these supernumeraries? There is no report of their participation to any of the adult tissue. It is therefore assumed that they die and become engulfed, maybe in the gut (Nardelli-Haefliger & Shankland, 1993; Weisblat et al., 1984). I investigated whether these cells were dying by programmed cell death (PCD) and if so, whether they were undergoing apoptosis.

The shape of the cells could be observed with lineage tracer (see Figure II.13). In all the lineages, the bandlet detaches from the germinal band. Two types of supernumerary cells were observed: the cells at the site of detachment are elongated, and disappear quickly; the cells posteriorly keep a cubic shape, stay organised in a bandlet structure and stay attached to the teloblast until it disappears, soon after detachment (e.g see Figure II.13 C before detachment, and D at detachment). The lineage tracer was sometimes seen to be leaking out of both type of supernumerary cells (as revealed by the lower intensity of the dye), revealing that the membrane may be damaged. However, this was not seen consistently.

Figure II.13 Dying supernumerary cells. (**A, B**): M lineage, (**C**): N lineage, (**D, E**): OP lineage.
(**A**) M labelled embryo, 73h after the birth of the M teloblast. The cells are detaching from each other, loosing their connection near the germinal band; they are also less bright, which suggests that their membrane might be degrading. The rest of the bandlet, more posterior (above the teloblast), is not visible at this angle.(**B**) M labelled embryo, 73h after the birth of the M teloblast. Both teloblasts have been injected. At this angle, both teloblasts are lying on top of each other. One one side, the cells near the germinal band are elongated whereas the cells close to the teloblast still form a cohesive bandlet. On the other side, the bandlet is twisted but has not yet begun elongating. (**C**) N labelled embryo, 88h after the N teloblast is born. The cells of the bandlets are still cohesive, but the cells seem vacuolated.(**D**) OP labelled embryo, 73h after the OP proteloblast is born. The bandlet has already detached, leaving the posterior end of both lineage at the same level (**E**) OP labelled embryo, 73h after the OP proteloblast is born. The cell closest to the teloblast is elongated, and the cells more anteriorly are also changing shape. The germinal band is not visible at this angle.
T: teloblast, B: bandlet, S: supernumerary cells

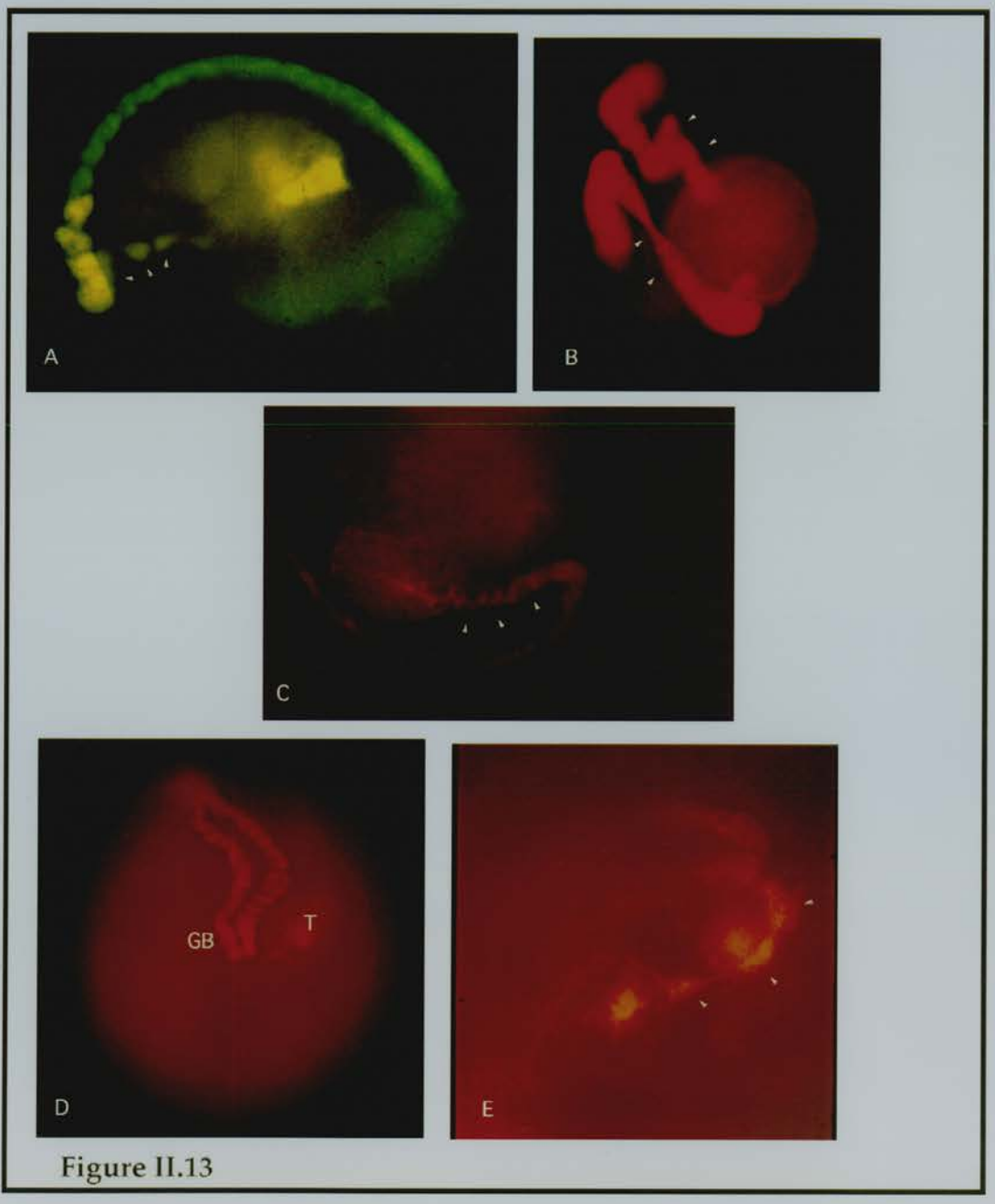

Figure II.13

The TUNEL method (in situ method for detecting overhanging single stranded DNA resulting from endonuclease digestion,(Gavrieli et al., 1992)), as well as labelling with the fluorescent chromatin marker Daunomycin were used to try to detect the condensation of the chromatin, but neither of these methods gave satisfactory results. I simply followed the supernumerary cells labelled through injection of the teloblast with fluorescent dextran. RDA was often taken up by the nucleus, and confocal imaging shows that the nuclei, even at the time they detach, do not present an alteration of the chromatin (see Figure II.14). Because not all the batches of RDA labelled the nuclei in the same manner, and the dye is not chromatin specific, I compared the staining obtained with the lineage tracer to the staining obtained with Hoechst. In both cases, the chromatin of the supernumerary cells was seen to resemble the chromatine of the older, segmental blast cells: there is no condensation of the chromatin, as long as the cells could be observed (a few hours after detachment).

Lucifer yellow was injected at different stages to detect whether the cells along the bandlet were connected by gap junctions until detachment. Lucifer Yellow is a small fluorescent compound, small enough to pass through gap junctions (Stewart, 1978). However, if the cells are not in a continous bandlet anymore, it could be expected that the dye would not travel between blast cells anymore. This was not found to be the case, even in later stages, a few hours before detachment (in the latest experiment, M was 64h old). The dye diffused into the whole embryo, to the neighbouring lineages and even to the countralateral side (See Figure II.15).

However, the Lucifer Yellow was not detected in between the cells, suggesting that the membranes had not become leaky either. Cells become leaky when they undergo necrosis or at the end of apoptosis (Dive, et al., 1992). Until M=64, the cells show no sign of necrosis or apoptosis.

Figure II.14 The nuclei of the supernumerary cells are not different from the nuclei of the segmental cells. Confocal image of a 70h old RDA labelled M bandlet. Same specimen as in Figure II.12. Anterior of the bandlet is to the top. (A) posterior part of the bandlet including both the segmental and the supernumerary blast cells. The bandlet has not yet detached from the teloblast even though the teloblast is not visible. The disruption in the bandlet happened after fixation, and the bandlet should therefor be considered continuous. Collection of 35 sections over 70µm. (B) The anterior, segmental part of the bandlet (collection of 6 sections over 12µm). The nuclei are represented in red. They are grouped as the cells are already visibly arranged into metameres. (C) The posterior, supernumerary part of the bandlet (collection of 6 sections over 12 µm). Here, single nuclei are visible, with the same intensity as the more anterior, deeper nuclei of the segmental bandlet (B). There is no evidence of nuclear condensation and apoptosis.
Scalebar is approximately 20µm.

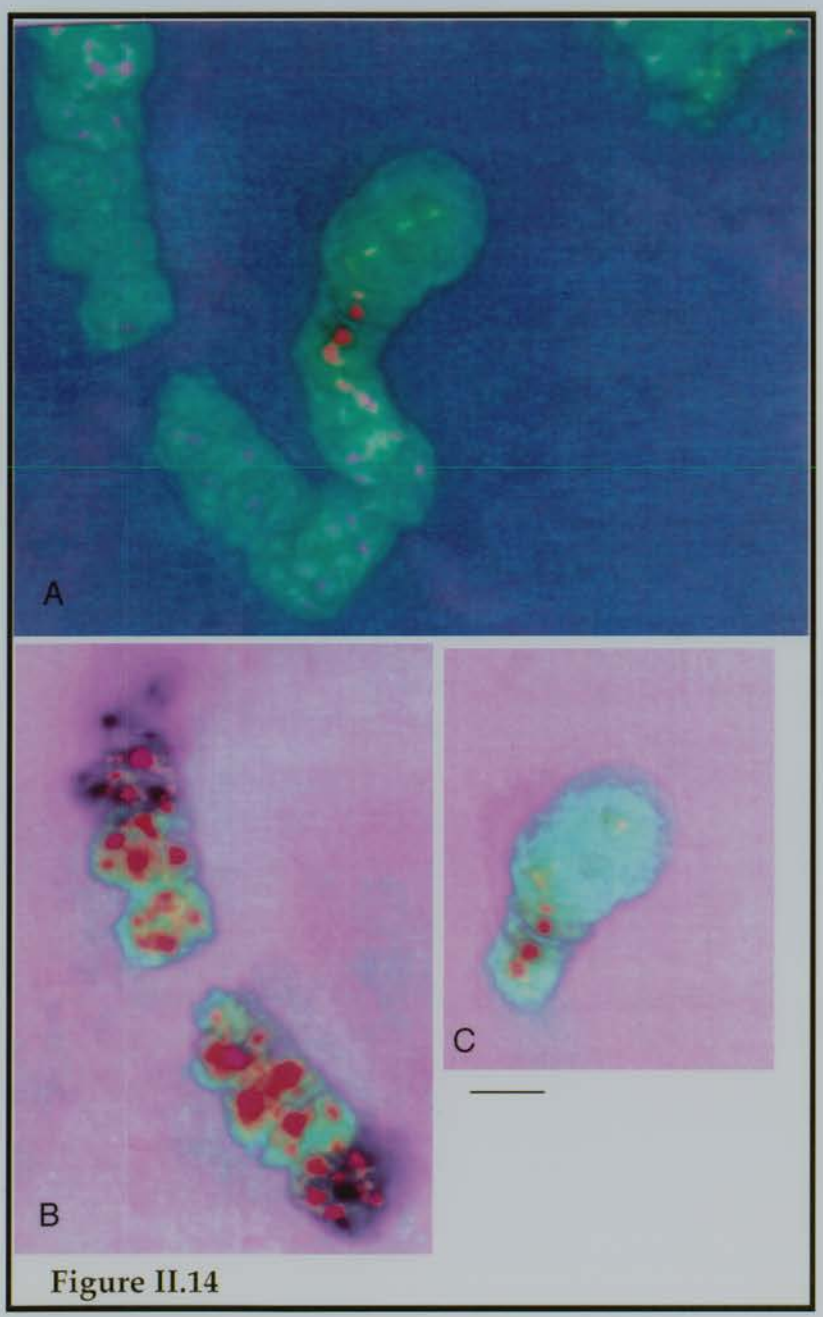

Figure II.14

Figure II.15 Results of Lucifer Yellow (LY) injection into the M teloblast. The M teloblast was injected with RDA and Lucifer Yellow. The embryos were viewed with a fluorescein filter. Lucifer Yellow appears yellow/green, and RDA appears slightly more orange, so that the originally injected teloblast could be detected. (A) embryo injected at stage 7 (when the M teloblast is still dividing). The dye infiltrates all the blast cells, including those from the neighbouring lineages. However, it does not seem to leak into the extracellular environment, as it is darker than the cells. Objective10x. (B) M was injected at M=40h, when M still divides, but to produce supernumerary cells. The injected teloblast is slightly orange, and the RDA stayed confined to the teloblast, but the Lucifer Yellow is in the whole of the germinal band and the other M teloblast, attesting that there are still gap junctions connecting the teloblast to the other cells, but that the teloblast is not leaking. Objective x25.

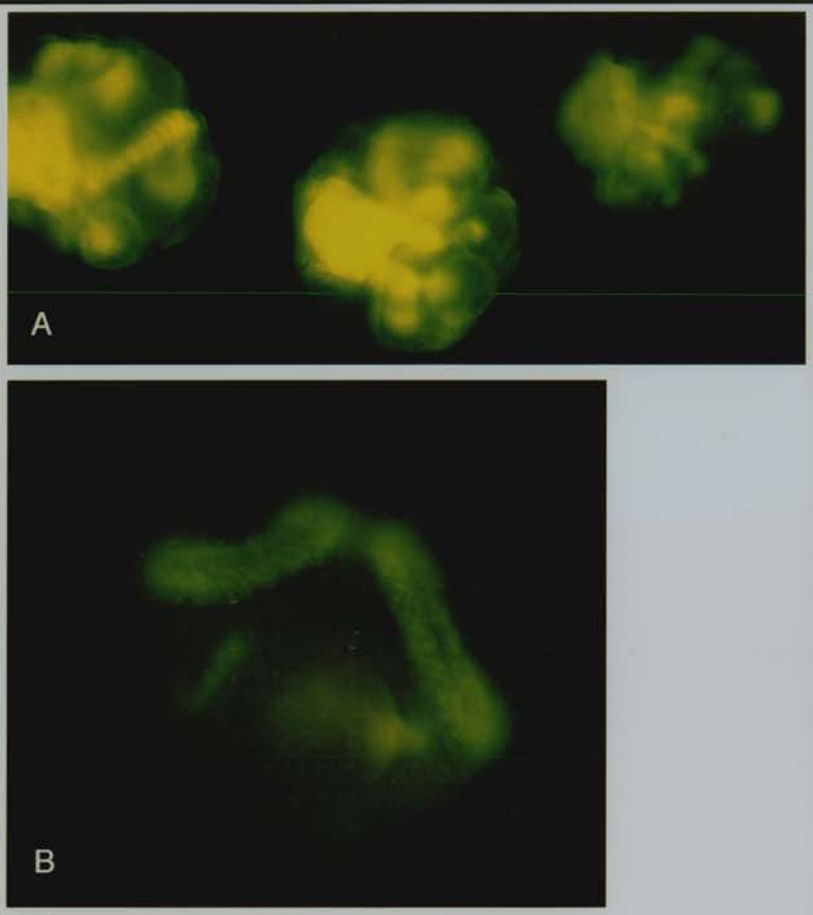

Figure II.15

Therefore, there are two types of supernumerary cells. The cells at the breaking point between single bandlet and germinal band and the supernumerary cells that stay in the single bandlet. Although the former were seen to undergo cell death, no positive sign of cell death was detected in the more posterior cells until they disappear soon after detachment. The main difference of these cells with the more anterior ones is that they do not divide so extensively.

Discussion

The result, a few years ago (Shankland, 1984), that some regulation of cell number could happen in the leech was very surprising: previously, in all instances where cells were ablated in the embryo, no regulation was seen to take place, and the cells were not replaced (Blair, 1982; Blair et al., 1990). It was previously assumed that the whole of leech development was cell-autonomous. In Shankland's experiment, a disruption of normal development made some cells change their fate to regulate the size of the germinal plate.

Shankland ablated cells in the O lineage at an early stage, in the germinal band. These ablated cells were not replaced. The ablation caused the bandlet to slip posteriorly, leaving it out of register with the other lineages. This had the effect that the cells of rank 30, 31 and 32 (when the bandlet was slipped by 3 segments) were now in register with supernumerary blast cells. As a result these cells adopted a supernumerary fate, and the O bandlet detached at the same level as the other bandlets (see for example Figure II.13, D). There was respecification of the fate of segmental blast cells. A seemingly contradictory result came out more recently (Nardelli-Haefliger, et al., 1994), when the same slippage experiment was used for testing whether the identity of a cell within a segment was cell-autonomous or position-dependent. This experiment used *lox2* , a segment identity gene of the HOM/Hox class as a marker of a subset of midbody segment. As a

result of slippage, the displaced cells retained the pattern of *lox2* expression according to their birth rank (see Figure II.16), rather than taking up the expression pattern of the new segment they were localised in. In this case, the segmental blast cells were not respecified.

Maybe the cells are not just fated to be in the germinal band and become segmental but require further signalling from the environment? In this chapter, I have investigated this possibility by observing the normal development of the leech (without disruption), looking for consistencies between embryos in their morphology that could account for a regulation of the size of the germinal bands.

The setting up of the limit between bandlet and germinal band

My results confirm the presence of supernumerary blast cells that do not go on to make segmental tissue, but detach from the bandlet and die (Fernández & Stent, 1980; Shankland, 1984; Torrence et al., 1989; Weisblat et al., 1984; Zackson, 1984; Zackson, 1982). These results also bring stronger evidence that the number of these supernumerary blast cells is not constant from one embryo to another, before detachment. Previously it had been suggested, once, that the number of blast cells detached "varied between 5 and 15" (Weisblat et al., 1984) .

Results confirm that the teloblasts undergo an unfixed number of cell divisions. This is a relatively counter-intuitive result considering that the leech is known to undergo a highly stereotypic pattern of cell divisions: there is to date no report of any other cell undergoing a variable pattern of cell division in the leech. Because the number of blast cells in the M and N bandlets is variable at the time of detachment (after the teloblast has finished dividing), the total number of cell divisions undergone by these teloblasts is not predetermined, and therefore the setting up of the posterior boundary cannot rely on proportionally dividing the total number of blast cells between segmental and supernumerary.

Figure II.16 Shankland's slippage experiment (Shankland, 1984 and Nardelli-Haefliger, et al., 1994;). see text for detail of the experiment.
(A) Cells can be laser ablated in the O lineage (here cells 7 and 8). (B) this results in the loss of these two cells and a delay in moving forward of the affected lineage. Here, the cell number 9 is in register with the cells from the segment 12. (C) the expression pattern of *lox2* is slipped accordingly. (D) But the pattern of cell death is unchanged, with cell death now affecting normally segmental cells that find themselves out of register.

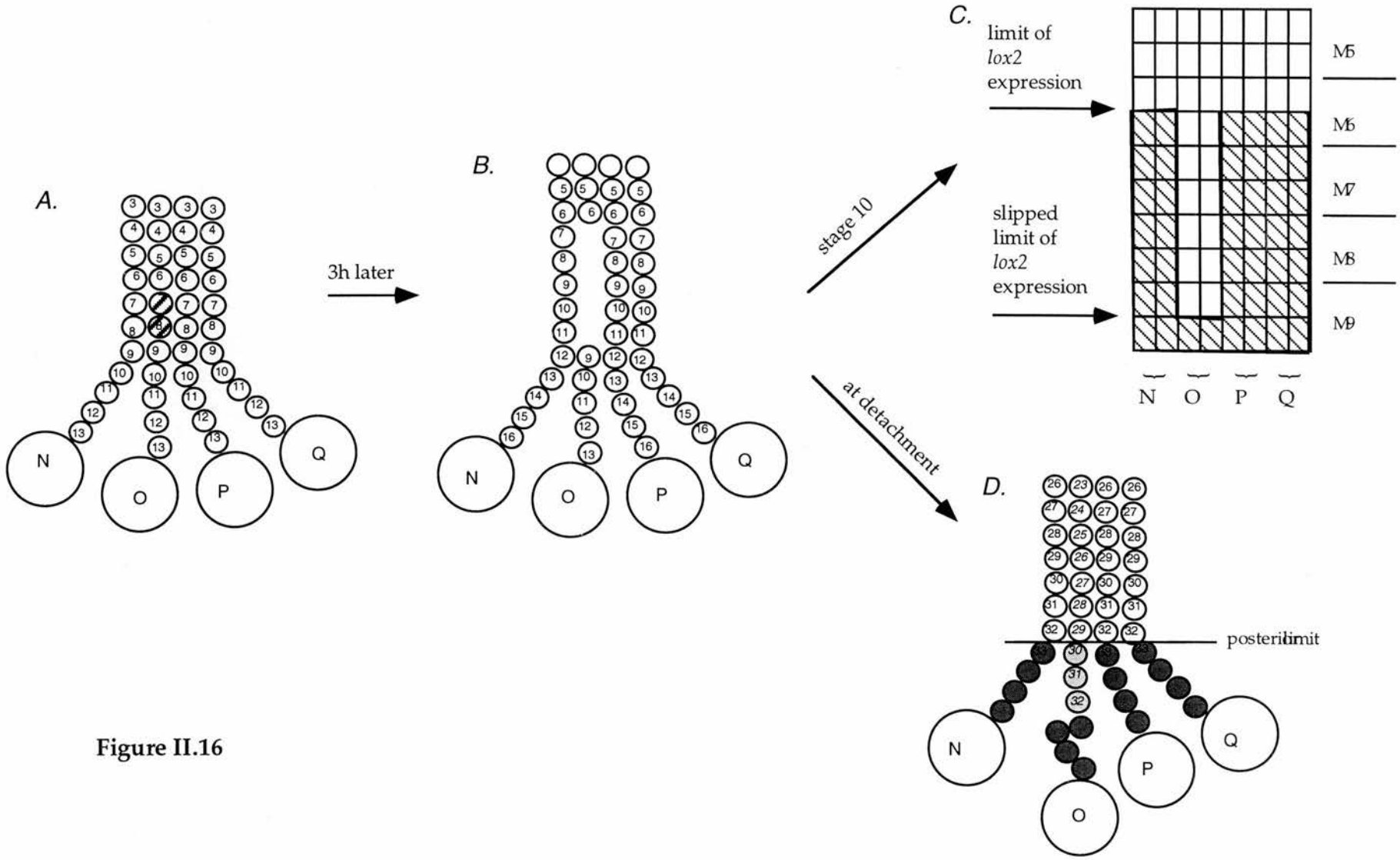
A.
3h later
B.
stage 10
C.
limit of
lox2
expression
slipped
limit of
lox2
expression
M5
M6
M7
M8
M9
N
O
P
Q
at detachment
D.
posterior limit

Figure II.16

The M teloblast divided at the rate of 1h15' throughout blast cell production, including supernumerary blast cell production. There is the possibility, however, that the teloblast has a resting period between the production of the segmental blast cells and the production of the supernumerary blast cells, at M=40h. This, however, would require further experiments to be confirmed.

The different lineages each detach at different time from the teloblast. This can be compared to the expression of segmentation genes (such as *engrailed (en)* and l*ox2*) which come on independently in the different lineages. *en* and *lox2* (Lans et al., 1993; Nardelli-Haefliger et al., 1994) are segment-specific genes, but they start their expression in specific cells before these cells are aligned with neighbours from the same segments: the segment identity and differentiation appears before the cells form coherent segments. Detachment, in the same way as segmentation gene expression, is not a concerted event: at the time the M bandlet detaches, the M lineages is not aligned with the other lineages, i.e. the blast cells of the different lineages are not yet in register; at the time O and P detach, their lineage is in register with M's but not with Q and N's; when Q and N detach, all the lineages are in register. Either all the lineages are totally independent from each other or one of the lineages could be setting the pace: M detaches first, then O and P around the same time, followed by N and Q much later. M could have its own regulation mechanism for detaching after 32 cells, and signal the end of the germinal band to the other lineages: when the time comes for them to detach, they are aligned with the M lineage. As it happens, the M lineage is the underlying lineage, the only one contacting all the other during the whole of segmentation.

By following the M bandlet in time, it has become apparent that the entry of the blast cells in the germband, and the connections consequently established (gap junctions) with the neighbouring bandlets might not be

required for the separation between the segmental and the supernumerary blast cells: the structure showed a too high variation from one embryo to the other.

These results provide additional information to the mechanism of regulation of the germinal band size. I have incorporated these results into a model that I propose here: the "is there anyone out there?" hypothesis.

As the blast cells are born, they acquire an identity (to translate into a specific phenotype later on) corresponding exactly to their rank of birth. This has been hypothesised previously (Shankland et al., 1991), and could be mediated by a variety of means. The simplest possibility is the dilution of a factor over each division, thereby providing each blast cell with a different cellular content. This identity would provide the blast cells with the capacity to differentiate according to their segmental position. This identity would also provide the difference between segmental and non segmental, or supernumerary, cells. The difference I suggest is one that provides only the segmental cells with a short-range system of communication, such as for example a short-range secreted or membrane-bound molecule, and its receptor. The role of these molecules would be to enhance the adhesion between the different bandlets in the germinal band, and communicate to the other cells "that they are in the germinal band". The supernumerary cells would not be able to produce either the signal nor the receptor, impeding at the same time the ability to form a germinal band and to communicate with each other (see Figure II.17). This signal needs to be very short range to accommodate the fact that cells slipped even as far as 1 segment cannot be incorporated into the germinal band.

In this view, the long timing between the time of last teloblast division and the time of detachment can be viewed as the time during which the blast cells can start expressing the signal and receptor. The detachment, however, is not a concerted event that happens in the different

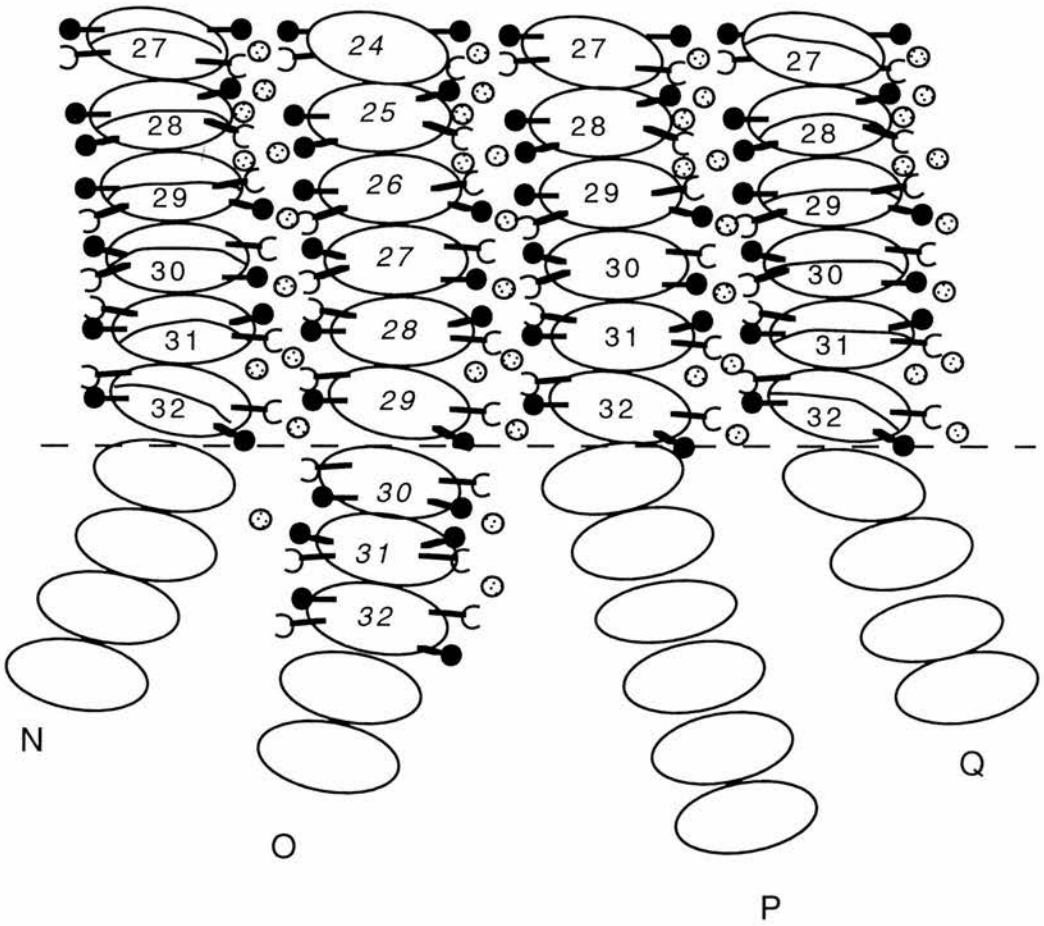

Figure II.17 "Is there anybody out there" model.
At birth, the cells acquire a segment identity later translated into a specific phenotype. The cells born before the 33rd round of cell division (i.e. cells 1 to 32 or 1 to 64 depending on the lineage) express a recognition pattern that allows them to make cohesion with their neighbours only if those also express the recognition pattern. For details of what the recognition pattern could be, see discussion and last chapter. The dotted line represents the cut off between the GB and the bandlets. In this drawing, the O bandlet has been slipped posteriorly. Although cells 30, 31 and 32 express the correct recognition pattern, they cannot communicate and make cohesion with their lateral neighbours because those do not express the recognition pattern since they are supernumerary.
In the N and Q lineage, the fact that two types of cells are produced per segment is represented by a line dividing each cell into 2.

Ligand, trans-membrane protein
Ligand, short distance diffusible molecule
Receptor

lineages at the same time. As they detach, each bandlet can only be in register with the older and already detached bandlets. In this model, we have to assume that M leads the way and knows where the limit of the germinal band is: at the time the M lineage detaches, the last N and Q blast cells are still being born (see Figure II.11). At the time the N (and Q) lineages detach however, they are in register, as observed by the fact that the line of detachment corresponded clearly with the germinal band limit in this lineage. Previous work is consistent with the idea that M might be "leading the way" in segmentation, for example, ablation of the M teloblast results in the loss of ectodermal segmental organisation (Blair, 1982).

The supernumerary cells

The study also aimed to characterise the phenotype of the supernumerary blast cells. Knowing when the cells become different and whether they follow a pathway known at the molecular level in another type of cell would help us understand what type of signalling is going on.

The earliest difference detected between supernumerary and segmental cells is the failure of the supernumerary cells to divide on time. This characteristic was not clear-cut, though, as some supernumerary cells were seen to divide near the border. We can interpret the result as follow: the teloblast, as it divides, distributes different molecules to the blast cells at birth. After 32 cell divisions, some components might be very low, such as cyclins. It is possible then, that the blast cells born after the 32nd do not carry enough cyclin to allow them to go any further than one or two cell divisions, or none for the most posterior blast cells. Alternatively, it might be that once detached, the blast cells can not divide any more, lacking some essential signal from the more anterior segmental cells.

The observation of the detachment of the bandlets through time also led to the surprising result that not all the supernumerary cells behave in the same way: the most anterior (closest to the germinal band) change shape

dramatically, becoming elongated, while the more posterior ones keep their cubical shape. Only the elongated cells disappear quickly, while the most posterior cells stay attached to the teloblasts, differing from the segmental blast cells only by the fact that they do not divide, and disappear all together later. I saw no direct evidence of their death. Of course, it is known that programmed cell death can be a very fast process of only a few minutes. However, this could happen up to 4h after detachment, and would be seen as a consequence of detachment rather than a means of size regulation.

The main characteristics of programmed cell death (PCD) are that it is initiated by a physiological signal and that it is part of the physiological process of development (Schwartz, et al., 1993). In the specimens observed, I always observed death of the cell at the junction between germinal band and single bandlet during detachment time, in a very short time-frame. The consistency of the pattern of cell death from one embryo to the other within a small time difference, suggests they follow a specific program, initiated by themselves or by an external signal. The characterisation of the supernumerary cells was aimed at finding out whether the program they follow could be related to PCD as has been described in the literature.

PCD is often described as a process where the nuclei undergo apoptosis, e.g. in the immature T-cells in the thymus (Schwartz et al., 1993). The ultrastructural changes include membrane blobbing (apoptotic bodies can be seen by scanning electron microscopy (SEM)), chromatin condensation (the chromatin becomes electron dense as seen by transmission electron microscopy (TEM)), and DNA fragmentation by endonucleases (this can be visualised by running the extracted DNA on an electrophoresis gel). In the case of the leech, not all these characteristics could easily be assessed, mainly because the cells concerned are few (this

makes any isolation of the cells and their DNA impossible) and are not on the surface of the embryo (this makes SEM impossible).

This phenotype was never observed in the more posterior supernumerary cells, and, as opposed to previous reports, are not seen to die. After detachment, their main characteristic is that they stop dividing. But their morphology stays similar to that of the more anterior ones. The fact that they dissappear could mean that their membrane fuses with that of the macromers, the cytoplasms mix, diluting any fluorescent lineage tracer, which becomes undetectable (Weisblat et al., 1984).

Conclusion

These results overrule the possibility that the development of the leech may be entirely cell-autonomous, in the view of the variability of the number of blast cells and the changeability of the fate of some of these blast cells in a perturbed environment. I propose a mechanism allowing for the regulation of the size of the germinal band involving local signalling.

The model could be tested in different ways. According to the model, segmental blast cells can be led to die by being placed in the supernumerary environment. This was shown to be true in Shankland's (1984) experiment. It also predicts that supernumerary cells cannot be rescued because of their phenotype from the time of birth not being sufficient to sustain them: I predict that supernumerary cells pushed forward in a segmental environment could not be rescued.

This model can also be distinguished from the antero-posterior gradient. This will be discussed in the final chapter.

Chapter III: supernumerary or segmental: where does the decision come from?

Introduction

In the previous chapter, I have shown that there is circumstantial evidence that the differentiation into the segmental or supernumerary pathway starts early on, before there is detachment of supernumerary cells from the more anterior germ band. At the same time, previous work (Shankland, 1984) suggests that the cells may not be commited to this fate, and that the environment may play a role in conserving the differentiation of the blast cells, and allowing them to become segmental or supernumerary.

Observations alone cannot distinguish whether a pathway is cell-autonomous or requires signalling from the neighbours. Techniques of experimental embryology can challenge the fate of a cell by modifying its environment; observing whether the same fate as in a normal environment is still followed will tell us whether any extracellular signalling is required for the pathway we are interested in. The techniques of experimental embryology available in the leech include cell ablation as the most important one. Cell ablation has been used to assess the role of specific cells on their neighbours (Blair, 1982; Blair et al., 1990). Ablation of some cells can induce transplantation of other cells, e.g. transplantation of blast cells by slippage of the bandlet (Shankland, 1984), another tool widely used in experimental embryology (change of the normal environment). However, isolation of cells has not been a very popular tool in leech embryology, presumably because the cells do not, like in other species, dissociate readily during dissection, even with the use of divalent-free medium and chelators. There is, however, one instance reported of such a culture, during the early

stages (Symes and Weisblat, 1992), and this is the technique I have used to study the regulation of the number of segments in the leech.

The signal received by the bandlet may come from the other bandlets, the micromeres, or the macromeres, or a combination of the above. To test the role of such an environment on the bandlets for the determination of which cells will become segmental and which cells will become supernumerary, I cultured single teloblasts from the time of birth and observed the type of blast cells produced.

In Chapter II of this thesis, I suggested that cell division was a satisfactory marker of the segmental blast cells since only segmental and not supernumerary blast cells divide extensively. In this experiment, I find that the blast cells produced in culture follow some of the divisions observed in the blast cells in vivo, but do not divide extensively.

Material and Methods

Culture

In the same way as for the injections, embryos were staged by clutch, and their development followed at 23°C. To isolate a chosen teloblast, embryos were dissected at a chosen stage, between 0.5h after birth of the teloblast and its further division. For the dissection, the embryos were transfered to a Petri dish in divalent ion-free medium (Calcium Magnesium Free medium, or CMF: as for the injections, Chapter II). To avoid the embryo sticking to the plastic, the Petri dish was covered with 1% agarose in CMF. For removing the vitelline membrane, sharp muscle needles were used. For isolating the teloblast, glass needles were used (long pulled electrodes) for applying pressure on the furrow between the cells. The cells were very rarely isolated without damaging the rest of the embryo, but it was often possible to isolate more than one teloblast from the same embryo. Once dissected, the explants were left to recover on 1% agarose, in Htr (see

Chapter II) with gentamycin (40 µg/ml), at 23°C. The cultures were observed at about 12h interval, and cultured for variable periods of time (see Results). They were fixed in 4% paraformaldehyde for about an hour. They could then be stained in different ways: for the nuclei with Hoechst (see Chapter II), and/or for cortical actin with FITC-Phalloidin.

Phalloidin staining:

Embryos were fixed for 1 to 2h in 4% paraformaldehyde in PBS. They were permeabilised for 1h using PBS-0.05% TritonX. They were stained for between 1h and 8h with Phalloidin-FITC (SIGMA) diluted 1/200. They were rinsed for a further hour and viewed under epifluorescence with a fluorescein filter, or using a confocal microscope.

Conditioned medium

A variable number of embryos (30 to 100) of the stage being cultured or of mixed stages were crushed in Htr medium (5 ml) and left at 4°C for a few hours before filtering, or filtered straight away through a 0.45µm filter.

Results

I isolated M teloblasts by dissecting leech embryos from stage 5, and cultured them using a variety of methods. The explants were left to develop and observed at different stages, and monitored for survival and division of the cells.

Optimisation of culture conditions

Many embryos were dissected, with different methods of dissection and culturing, until a high rate of survival and cell division could be recorded.

The cells were difficult to dissociate from each other, and many embryos had to be dissected to get a few teloblasts to survive in culture for the desired length of time: explants were fragile and prone to infection in

the same way that devitellinised embryos are. In some of the batches, none of the isolates survived or divided. In others, a fairly good proportion survived. In the batches with high survival rate, I would expect 10 to 15 correctly dissected from a clutch of 20 embryos, out of which 5 to 7 would survive and divide. Some observation could be done under the dissecting microscope: alive/dead or bandlet/no bandlet phenotypes could be distinguished. A pink rather than white coloration, and no hollow parts suggested that the cell was still alive. A clear pole or a clear line in the explant suggested that the teloblast had produced a bandlet. However, individual cells were not counted in this part of the experiment, and the bandlet was not always obvious.

The method of dissection affects the survival of the cells in culture

Cells dissected rapidly from the rest of the embryo tended to start looking unhealthy rapidly, presumably due to damaging of the membrane. The embryos were therefore dissected by exerting continuous pressure on the intercellular furrow (Symes & Weisblat, 1992) for one or two minutes, using clean glass needles that didn't stick to the embryo.

The cells cannot adhere to a substrate on the culture plate

Different plating methods were used to assess whether the cells required to adhere to a substrate or not for division. When plated on tissue culture plastic, the cells did adhere, but this seemed to kill them. Of the healthy cells recovered, none was attached to the bottom of the well. Therefore, in order to prevent sticking to the plastic, the wells were coated with agarose made up using the medium the cells were cultured in.

To mimick the pressure exerted on the cell by the rest of the embryo, some cells were placed in microwells, formed on the agarose layer with a hot needle. This did not affect the survival and division of the cells in culture and was not retained.

The cells do not need a complemented medium

The basic medium used for the cultures of the single cells was the same medium as for the culture of whole embryos, Htr (Blair and Weisblat, 1984). Different supplements were used such as foetal calf serum, or more simply embryonic extract from leeches of the same stages (conditioned medium). These did not affect the rate of survival or the number of explants dividing. Therefore, the explants were cultured in Htr medium, with no other addition than antibiotics.

The M bandlet develops outside the embryonic environment

Because the explants were fragile (they would "explode" at the air-water interface), I had to fix them and stain them to observe them further by mounting them. In this process, more explants would be lost. The results presented below therefore come from a limited number of observations. One batch of embryos (between 15 and 30 embryos) would only allow a maximum of about 8 cultures to be set up. Of these cultures, not all were healthy from the begining (due to a too rough dissection), and often only 2 or 3 cultures reached the end of the culture time (24 to 72h). 24 independent experiments that yielded divided teloblasts in culture are reported here (see Table III.1). The information that could be gathered about each culture depended on whether it could be fixed or had to be observed live (in order to observe it again later); Figure III.1 shows the difference between the main types of observation of a typical explant.

Only the M culture was attempted: at stage 5, the M_R (right teloblast) is a cell that can be relatively easily isolated by virtue of its position in the embryo (see diagram of leech devlopment, Chapter I). Different experiments were done, sometimes isolating the M cell only, sometimes isolating the M cell with the underlying macromere (for providing a support for bandlet crawling; or allowing actual communication between the cells), sometimes the 2 M cells together (to see the relation of 2 bandlets

table III. 1

Under 24 h

nº	stage	cell(s) extracted	age of culture	means of viewing	Result/ Observation
1	4c	M(12)	4h	compound (live)	all healthy but no division
2	4c	M (1)	7 to 19h	compound (video)	1 equal division observed (at 11h of culture) before dying
3	4c	M + macromere (1)	1h30 to 11h	compound (video)	3 blast cells at least
4	4c	M (2)	12h	dissecting microscope	1 equal divison
5	4c	M + macromere (1)	12h	dissection microscope	pole of blast cells
6	4c	M (3)	12h	dissection microscope	pole of blast cells
7	5	M (1)	12h	dissection microscope	cannot see obvious blast cells
8	6a	M + macromere (2)	12h	dissection microscope	1 seems to have blast cells
9	5	M (6)	14h	dissecting microscope	pole of blast cells
10	4c	M (9)	18h	compound (video)	most cultures have between 2 and 4 equal size cells
11	4c	M + macromere (2)	18h	Hoescht (live)	approximately 6 cells in a bandlet, (Figure III.2)
12	4c	M (6)	19h	dissecting microscope	4 cultures divided equally once, 2 are alive, undivided
13	5	M (6)	19h	dissecting microscope	pole of blast cells

24 to 70 h

nº	stage	cell(s) extracted	age of culture	means of viewing	Result/ Observation
14	4c	M (2)	24h	dissecting microscope	pole of blast cells
15	4c	M + macromere (2)	24h	dissecting microscope	pole of blast cells
16	4c	M (1)	29h	fixed (H + P)	10 to 20 nuclei, concentrated.
17	4c	M (5)	31h	dissecting microscope	3 to 5 have pole of blast cells
7	5	M (1)	36h	fixed (H + P)	14 blast cells (Figure III.5)
8	6a	M +macromere (1)	36h	fixed (H + P)	bandlet (number of cells undetermined)
18	4c	M +macromere (2)	39h	fixed (H + P)	1 culture has a bandlet; 1 culture has many large cells
8	5	M (6)	40h	dissecting microscope	pole of blast cells
19	4c	M (2)	43h	fixed (H + P)	The nuclei are visible but there may be no organisation in bandlets
20	6a	M (1)	48h	fixed (H + P)	pole of blast cells at the anterior (Figure III.6)
21	6a	2M	48h	fixed (H + P)	1 bandlet that runs between the 2 teloblasts (Figure III.7)
11	5	M (6)	48h	dissecting microscope	pole of blast cells
22	5	M (4)	48h	dissecting microscope	pole of blast cells
23	4b	DM/DNOPQ	48h	fixed (H)	segments (see Figure III.8)

Over 70h

nº	stage	cell(s) extracted	age of culture	means of viewing	Result/ Observation
2	5	M (4)	72h	dissecting microscope	bandlets
4	5	M (3)	72h	Confocal (P+D)	21 nuclei in bandlet, 1ry and 2ry (figure III.9) pole of cells (no organisation) 1 is alive, undivided

able III. 1 Results from 24 independent culture experiments. The results are classifed according to the age of the culture at ıe time of observation into 3 different groups. When the same experiment was observed more than once, the same entry ımber is used twice (e.g. culture 7 and 11). The embryos were dissected between stage 4c and 6a (column 2 is the stage of ssection). In the column 3, is the type of cells that were put in culture as single explants, and in brackets the number of such ıltures for each experiments (only the ones that survived to the end of the experiment are recorded). Column 4 (culture) is the ge of the culture. The way the culture was viewed (live/fixed and optics used) is recorded in column 5.
: Hoechst stained; P: Phalloidin stained; D: Daunomycin stained

Figure III. 1 Example of 48h in culture of a proteloblast. Different cultures viewed live under the dissecting microscope (A), fixed, at 25x magnification (B), and fixed, at 40x magnification, using fluorescence to reveal Hoechst (C) . In A, it is not possible to tell whether the explant has produced blast cells or not. In B, the proteloblast has divided into teloblasts (black arrows); and these have been producing blast cells (white arrow). In this example, the cells have changed their relative position. The blast cells have converged but without staining it is impossible to determine if they form further bandlets. This locallised cell growth was sometimes also observed in single teloblast explants (see figure III.6). In C, it is possible to see that there are blast cells (nuclei stained blue), and that they are arranged into bandlets (white arrows point to the nuclei).

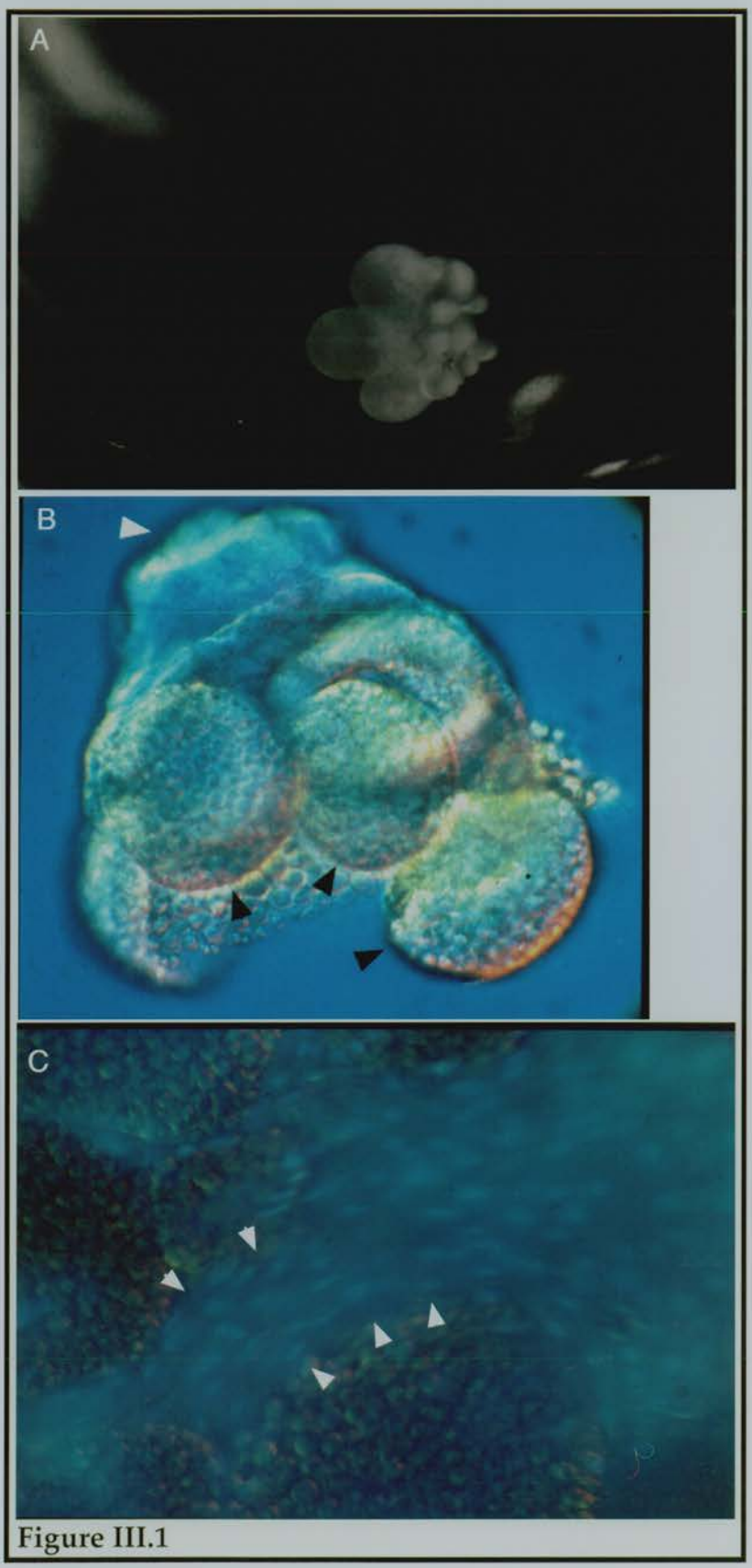

Figure III.1

of the same type), and sometimes the 2 M cells and macromere, the closest to an NOPQ ablation (refer to Figure I.2 and table I.2, Chapter I). An NOPQ teloblast would sometimes be isolated as a control of the dissection (it is easier to see if division has been going on because of the multiple teloblasts produced before bandlets). I also cultured the explants for different times: under 24h to see whether blast cells are produced or not; between 24 and 70 hours to see whether an organised bandlet is formed; and over 70h to see whether 2 types of blast cells (segmental and supernumerary) can be observed as in vivo at that stage (see Chapter II for timing in the bandlet; Figure II. 11)

In the case of bandlets being produced, I looked at the morphology of the cells and compared them with the morphology of the cells produced in vivo. I have described in the previous chapter how I found that segmental and supernumerary cells could be be distinguished morphologically, at least after a relatively late stage (after 60h for the M lineage, i.e. middle stage 8), but before separation from the germinal band; I have looked at the same criteria of cell division and cell death in the cultures.

Under 24h cultures

None of the 12 M teloblasts cultured for 4h showed any sign of cell division (see Table III.1, experiment n° 1). Cultures left for longer, however, often divided, in one of two ways: equally or unequally. If the teloblast had divided equally (in 15 cultures from 4 independent experiments, e.g. experiments n° 2, 4, 10, 12, Table III.1), the resulting cells were not seen to further divide unequally (produce blast cells). In cultures where teloblasts dividedunequally, blast cells were identified under the dissecting microscope as a pole of transluscent cells. These poles were observed in 18 cultures from 7 independent experiments (experiments n° 3, 5, 6, 8, 9, 11, 13). Only in one experiment the cultures were observed under epi-fluorescence, and the number of blast cells counted (experiment n° 11, Table

III.1 and Figure III.2). A minimum of 6 blast cells had been born during an 18h culture, a number far smaller than that expected from a teloblast in vivo (approximately 15 blast cells). These cells were organised in a bandlet in both cultures.

Some cells were followed by video to try to observe the onset of cell division (experiment n° 3 and 10, Table III.1). However, maybe due to the fact that the cultures had to be mounted beween slide and coverslip for better observation, the cultures never survived as long as their counterparts cultured in petri dishes. One such video showed an unequal division 11h after dissection of the teloblast, and another showed 3 blast cells, 11h after dissection of the teloblast, suggesting 3 divisions had occured. However, these divisions were not observed because they had not occured in the field of view of the camera.

Between 24 and 70h in culture: cell cycle of the blast cells in culture

Control embryos fixed and observed 24h after birth of the M teloblast show that the bandlets of the different lineages are already joined in germinal bands (see Figure III.3).

In this window of time, the teloblasts in culture had either divided unequally, forming a pole of blast cells, or had degenerated. Only 1 teloblast after 39 hours showed many large cells rather than one large and many small cells (experiment 18, Table III.1). This particular culture did not present a pole of blast cells. A few control cultures were done, of an OPQ cell for 36h and of an NOPQ cell for 48h (see Figure III.4). These presented 3 to 4 large cells and a pole of blast cells, suggesting that it was probably due to the type of cell rather than the culture conditions that equally divided M teloblast were never seen to make blast cells.

In explants cultured between 24 and 70h, fewer blast cells than in vivo were recorded. In 2 different explants, I observed mitotic figures among the blast cells produced, resulting in primary blast cells (born from

Figure III.2 M explants after 18h in culture, both from experiment n°11 (see Table III.1), live. A and B: darkfield and fluorescence of the same explant respectively. C, and D: bright field and fluorescence of the same explant arrows point to the cells born in culture, forming a bandlet. The fact that The Hoechst staining reveals nuclei on the surface, but also nuclei deeper in the yolk (macromeres) that could not be observed with only incident light.

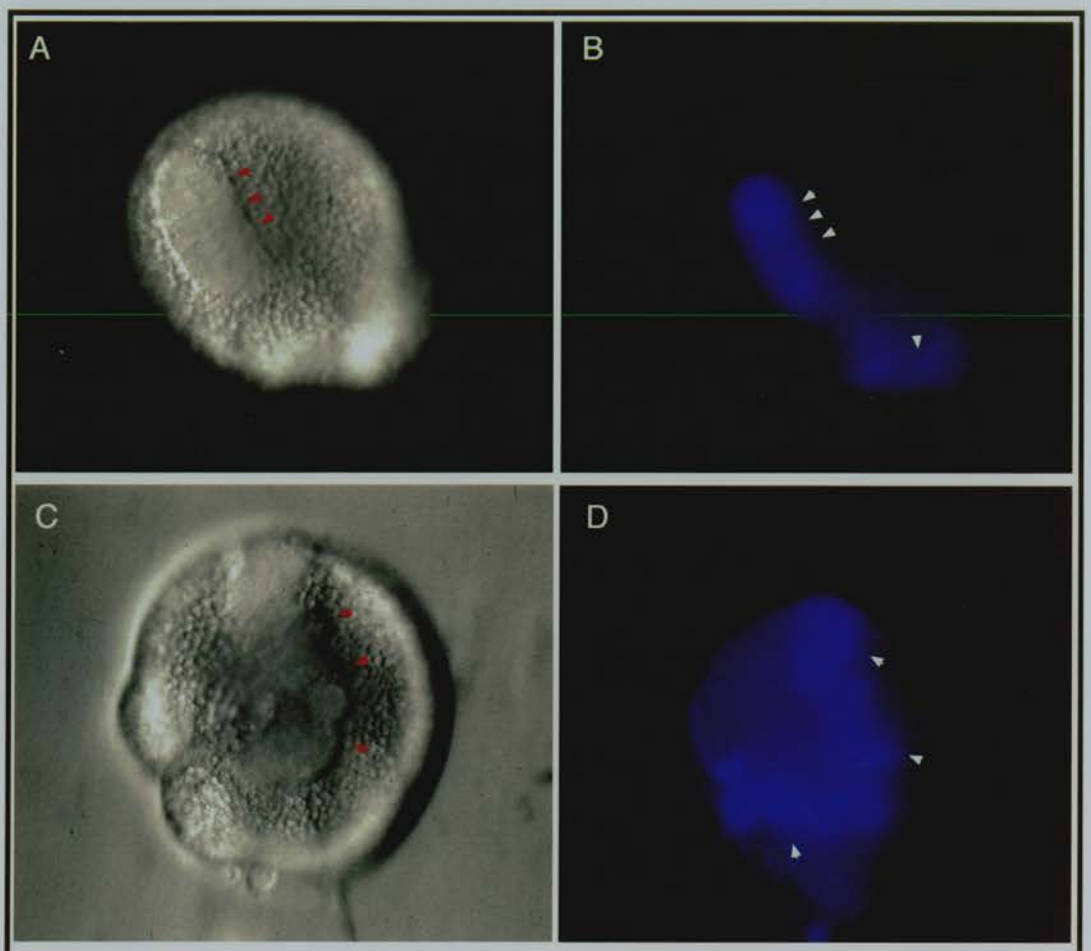

Figure III.2

Figure III. 3 Control. 24h after birth of the M teloblast, middle stage 7. Posterior view: the bandlets have converged into the right and the left germinal band. (a) phalloidin staining, (b) Hoescht staining. (c) is a high contrast of (a). The black arrows point to the teloblast, the white arrows to the left and right germinal bands. Magnification x25, scalebar: approximately 100 μm.

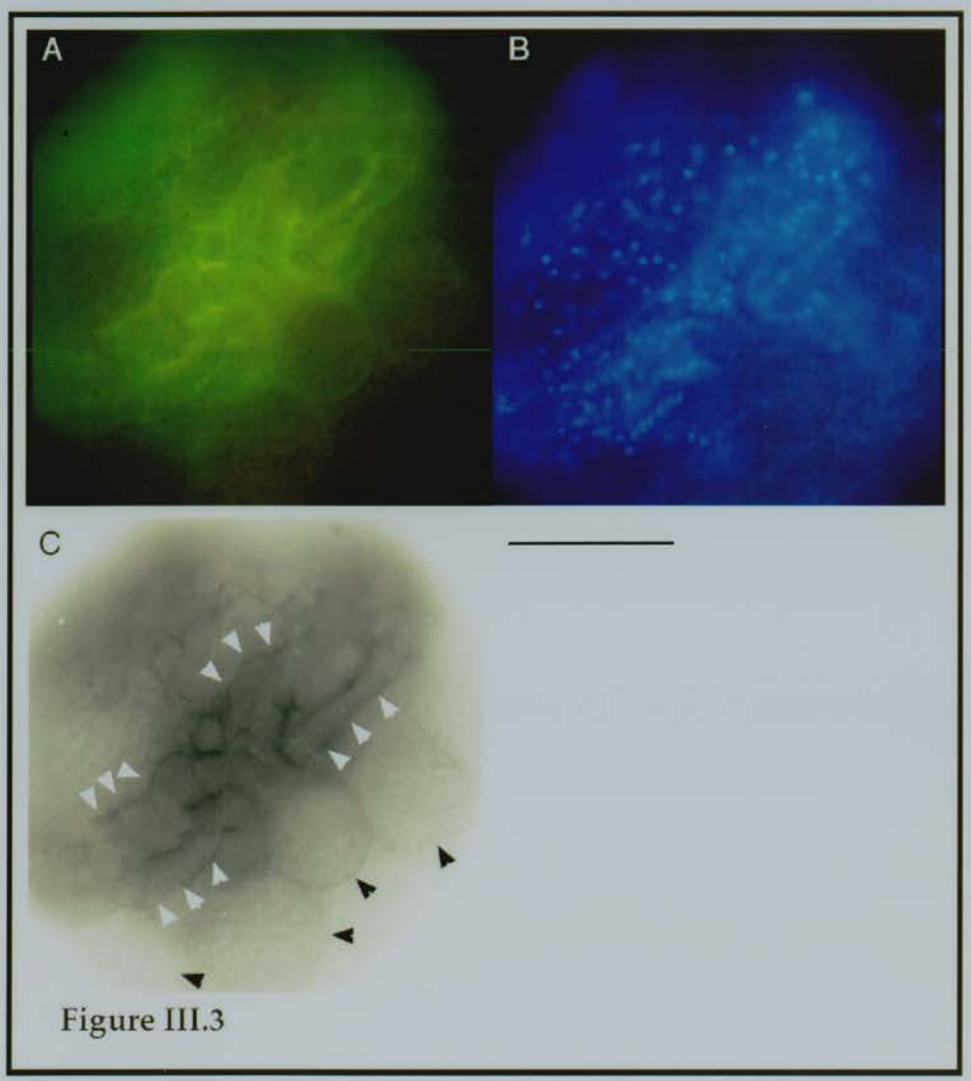

Figure III.3

the teloblast) and secondary blast cells (born from the division of a primary blast cell). In both cases I was able to count the number of cells in the bandlet (see Figure III.5). The pattern of division resembled that observed in vivo: a division was observed 8 cells anteriorly to the teloblast, and a further division (of the secondary blast cell) 5 cells ahead. If the cell cycle of the blast cells is conserved, this means, like in vivo, a cell cycle of 8h for the primary blast cell and a cell cycle of 5h for the secondary blast cell (Bissen & Weisblat, 1989) However, I never attempted to record the actual time of division, using BrdU for example.

Once the culture had grown to produce many blast cells that in turn start dividing, it became difficult do ascertain whether the cells were organised in a bandlet anymore, how many cells were produced, and which of the primary blast cells had divided to form secondary blast cells (e.g. experiment 19 cultured for 43h and experiment 20, cultured for 48h, see Figure III.6). When 2 M teloblasts (from the same embryo) were cultured together for 48h, a pole of cells wasn't observed but two distinct bandlets had formed, each on one side of the explant (experiment 21, Figure III.7).

A control culture of the DM and DNOPQ was done to find out the relative role of the micromeres and the macromeres on the division of the blast cells in culture and their organisation into bandlets. The 48h culture was organised into a structure ressembeling the germional plate, and showing sign s of segmentation (see Figure III.8)

No difference in the number of blast cells was observed between the bandlets that had grown from a teloblast and those that had grown from a teloblast cultured with a macromere (e.g compare cultures 8 and 18 with culture 7). However, the explants containing a macromere formed bandlets rather than disorganised poles of blast cells.

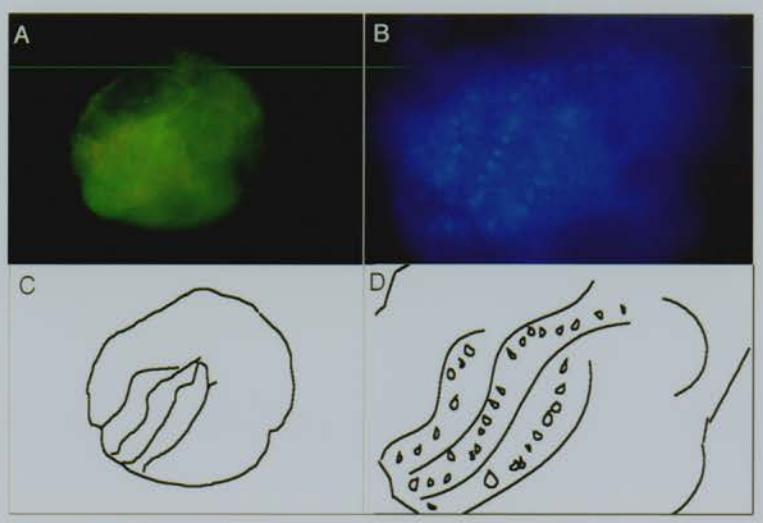

Figure III.4

Figure III.4 Control cultures. OPQ explant after 36h. The proteloblast has divided to give rise to 3 teloblasts producing bandlets. The bandlets converge as in vivo. The OPQ explants were not scored for division of the blast cells because of their longer cell cycle in vivo. (A) Phalloidin staining, (B), Hoechst staining. (C, D) line drawing of a and b, outlining the bandlets and (in D), the nuclei of the blast cells.

Figure III. 5 M explants after 36h in culture (magnification x40, scalebar approximately 100µm), black arrows point to nuclei that have just divided.
(A), (B) and (C) are different views of the same explant; the blast cells have had time to divide twice. (B) posterior is at the bottom; 8 cells can be counted posterior to the first dividing cell, (A) anteriorly 4 pairs of cells can be counted between the two dividing cells (C) most anterior part of the bandlet(to the left); 12 cells (coming from 3 founder blast cells that have divided twice) can be counted anterior to the most anterior cell division, with no obvious sign of further cell division. This makes the total of cell divisions undergone by the teloblast 8 + 5 + 3 = 16.
(D) In this explant, a total of 20 cells were counted, out of which 8 were undivided, and 6 had divided once. The teloblast had divided 14 times.

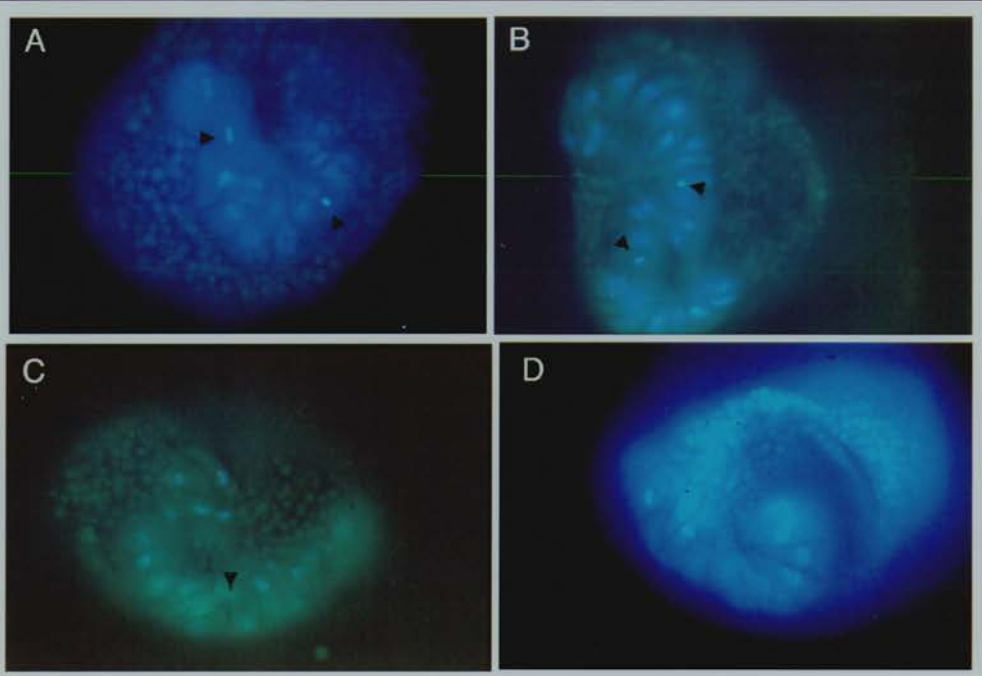

Figure III.5

Figure III. 6 M explants in culture after 48h (exp. n° 20). A: Phalloidin staining alone, B: Phalloidin and Hoechst staining, double exposure. Scalebar: approximatly 100 µm

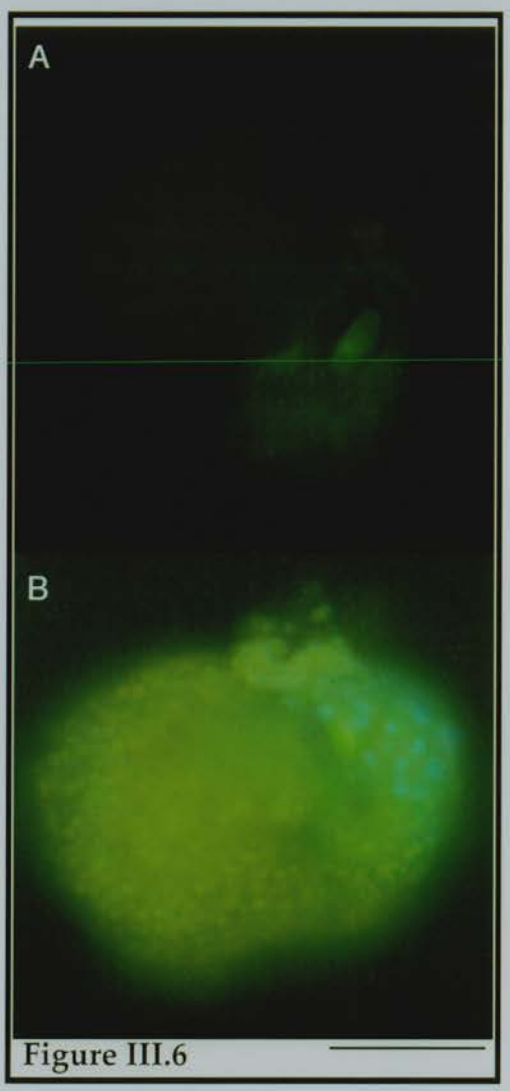

Figure III.6

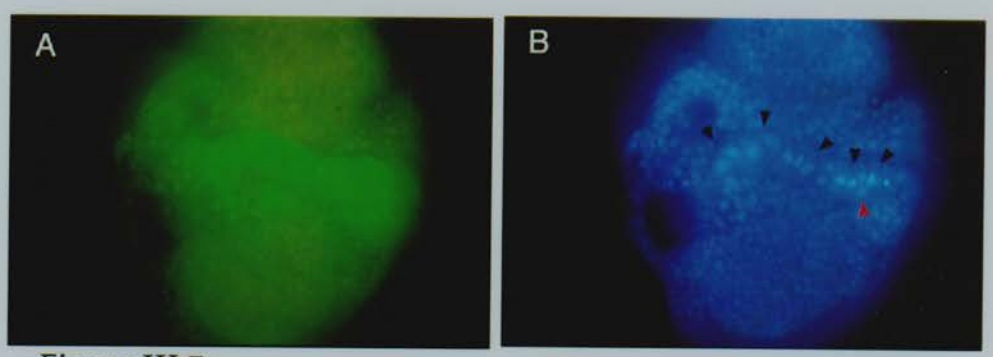

Figure III.7

Figure III. 7 2M explant, after 48h in culture. Only one of the 2 bandlets is visible, the other one is on the other side: the two don't converge. (a) phalloidin staining, showing the shape of the cells (b) Hoescht staining. The black arrow points to the nuclei of the cells organised in a bandlet. The red arrow points to the nuclei that have just divided.

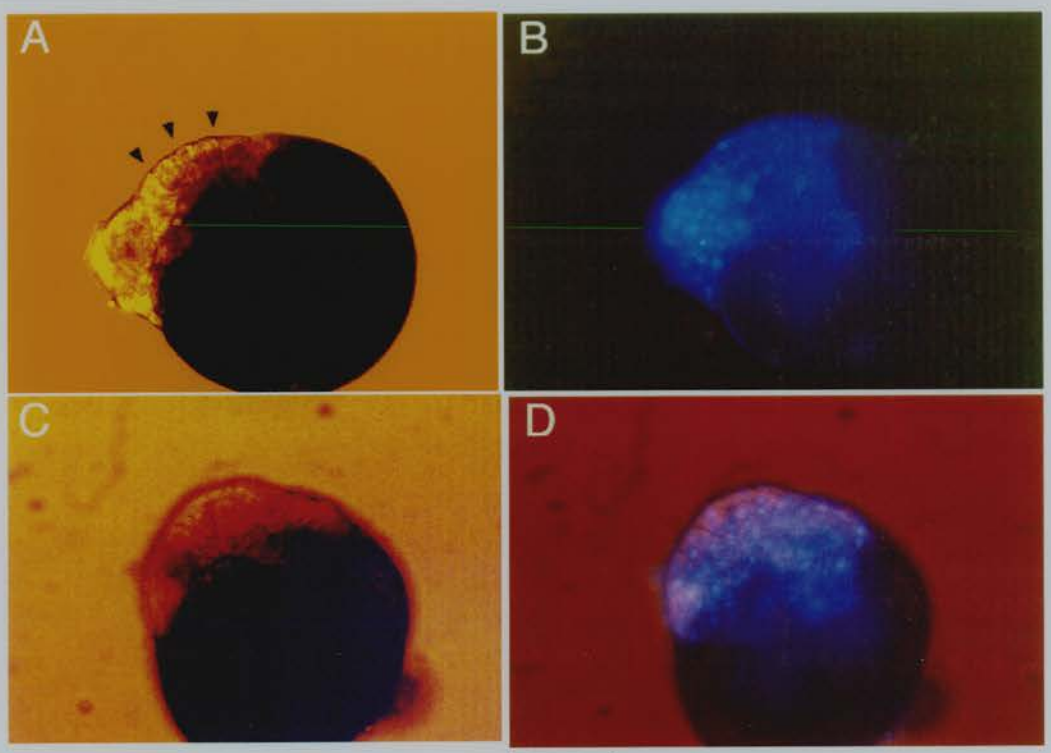

Figure III.8

Figure III. 8 Control cultures. DM / DNOPQ explant (see experiment n°23, Table III.1) after 48h. Some organisation, such as segments (arrows), are visible among the transparent cells produced. A: bright field, B: Fluorescence (Hoechst staining); C and D: another explant from experiment 23, this time showing no organisation. Scalebar: approximatly 100 µm

More than 70h in culture

Explants survived in culture for at least 72 h , producing more blast cells and more secondary blast cells than explants of 50h, as expected (see Table III.1). For that reason it was difficult to count the exact number of blast cells, and distinguish between primary, secondary and maybe tertiary blast cells. However, mitotic figures in the blast cells could still be seen and there was no observation of cell death or bandlet detachment comparable to what is observed in the control at that stage (see Chapter II), as observed both with the morphology of the nucleus (with Hoechst) and with the morphology of the membrane (with FITC-phalloidin), see Figure III.9. Again, both disorganised poles of blast cells and organised bandlets were observed. In case of an organised bandlet being formed however, no metameric structure was observed. This is in contrast with the M lineage in vivo where the cells are organised in repeated structures from mid-stage 8.

Discussion

As in other species, Xenopus, (Cooke and Webber, 1985), ascidians, (Reverberi and Ortolani, 1962), sea urchins (Wilt, 1987) etc..., blastomeres of the leech can be isolated from the rest of the embryo and maintained on their own in culture with no supplementation: the cells are self sufficient to a certain extent. This characteristic is specific to blastomeres over any other cell : cells (adult) always require other cells or supplemented medium to maintain themselves (Raff, 1992). In the case of the M teloblast in culture, the cell stays alone in culture for up to 20h, after which time it divides. From then on, signalling can occur between cells. The fact that cells can be maintained in culture without any supplementation only suggests that no signal is required for their survival. It makes no assumption about whether any signal is required for allowing them to follow their fate.

Here, I have looked at the cell division of the M teloblast (a stem cell), and of its descendents, the blast cells: in this experiment, I have isolated an

Figure III.9 Confocal image of M teloblasts in culture for 72h. The explants were stained with phalloidin to highlight the cell membrane. However, due to the accumulation of cells at one pole, it is impossible to count the number of cells or detect whether they are organised in a bandlet. (A) was definitely organised in a bandlet, but (B) was disorganised.

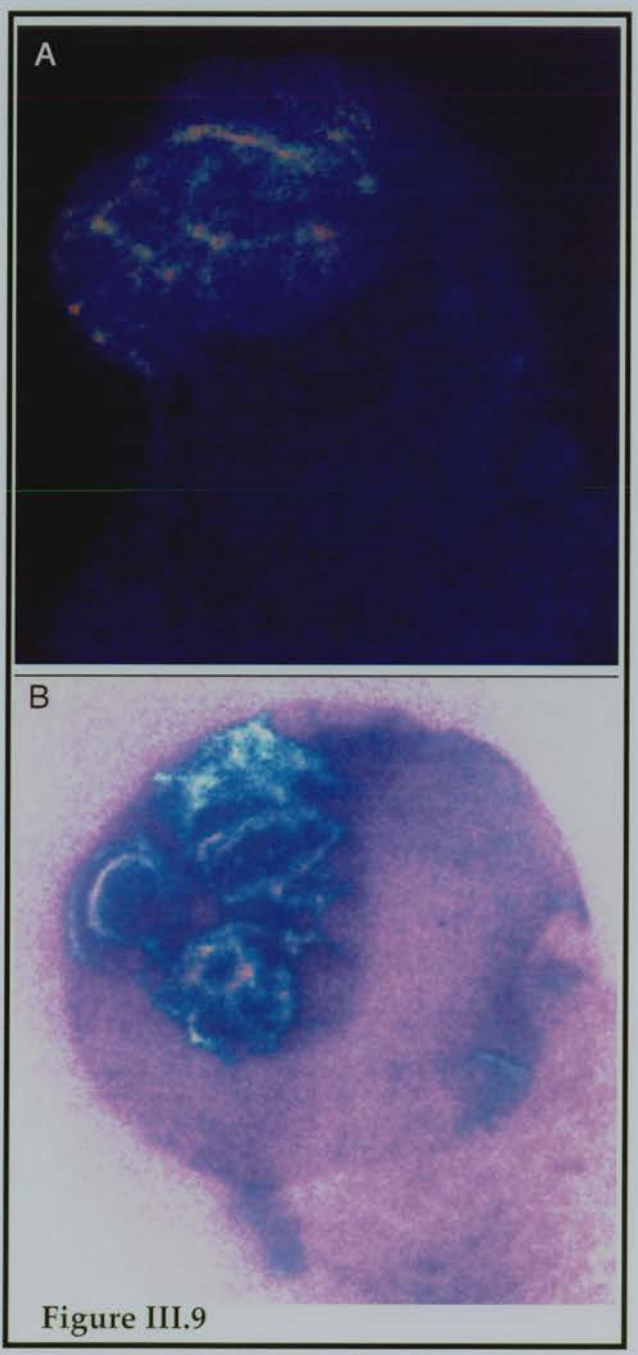

Figure III.9

M teloblast and its progeny from the rest of the embryo by means of culture, starting with the teloblast alone, and looked at the division of the teloblast and its descendents.

Among the teloblasts that divided, different behaviours were observed, such as undetermined general growth localised at one pole of the explant or equal cleavage; alternatively, I observed bandlets with different degrees of resemblance to the in vivo bandlets. Because the proportions between these different behaviours were not consistent from one experiment to the other, different phenotypes were attributed to inherent differences between explants, that could not be controlled (e.g. trauma during dissection, time it takes for the dissection, stage of the cell cycle the cell was in at the time of dissection, pressure exerted on the explant during cell division).

Since no ectoteloblast (N, O/P, or Q) was present in the explants, germinal bands could never be formed (the M teloblasts were always observed to divide directly into blast cells, therefore one cultured teloblast always gave rise to only 1 bandlet). Even in the cases of two M teloblasts being isolated in the same explant, the bandlets did not converge (see Figure III. 7). Comparing the explants coming from 2 M teloblasts (Figure III. 3) and from 1 NOPQ proteloblast (Figure III.4), I can conclude that the bandlets do not just merge with any other bandlets to form the germinal band: the bandlets in the NOPQ explant converge (as expected in vivo, see figure III.3), whereas the bandlets of the 2 M explant don't. The bandlets need to recognise that the other bandlets are different from it before merging into the germinal band.

In a large proportion of these cultures, the teloblast undergoes a stem-cell-like division, and the blast cells produced can arrange themselves into a column, or bandlet of cells as observed in vivo. Differences between the cultures and the in vivo M lineage were observed, mainly that the

number of blast cells was smaller than in vivo; the blast cells started following cell divisions in a similar pattern to in vivo, but did not form somites (the blast cells in vivo give rise to clones of approximately 10^2 descendents, (Weisblat & Shankland, 1985)); and no supernumerary cell morphology was observed.

Fewer cells are produced by the teloblast in culture than in the controls. This can be interpreted in different ways: (i) the cell cycle of the teloblast may altered, maybe lasting longer, or (ii) there may be a lag in the starting of the teloblast division due to the trauma of the dissection. Such as lag is known to occur in injections (1h reportedly), or (iii) there may be both a lag and a modification of the cell cycle. Because no divisions were seen in 4h cultures, I concluded that it was most likely that there was an important lag in the begining of the explanted teloblast cell cycle. However, this lag could be variable in time since some cultures had already produced a bandlet after less than 24h, and some produced no detectable blast cells in 24h but a perfect bandlet in 36h. Because of these discrepancies, the time of the cell cycle of the teloblast in culture could not be measured. The number of blast cells produced could not be predicted from the length of the culture. Some explants formed poles of cells (rather than bandlets) earlier than others. These poles could be due to an inorganisation of the undivided blast cells, or to more blast cells and their descendents having been born (compare the cultures of 18h, 36h, 48h and 78h, Fig. III.2, , 5, 6, 7 and 9).

The blast cells were found to follow neither a segmental fate (they do not make somites), nor the fate of the intermediate supernumerary cells (they do not die). Previous experiments are consistent with this result (Blair, 1982): when the ectoteloblast precursor (NOPQ) is deleted, the M lineage does not form somites. In vivo, the descendents of one m blast cell populates 3 different segments (Weisblat & Shankland, 1985). It is possible that the segments cannot be formed because the blast cells cannot "mingle"

in with the other lineages. This could also explain the pole of cells observed in older cultures (48h). It is possible that the M blast cell descendents actively migrate in order to find their place in the segments; the absence of a large majority of cells forbids them to to move, and results in all the cells "piling up" on top of each other. However, the blast cells in culture did undergo cell division in a pattern reminiscent of the first 2 divisions of blast cells in vivo. The cells are capable of dividing, maybe even following a predetermined program of cell division, but only to a certain point; we have seen also that, in vivo, although the majority of the supernumerary cells do not divide, the most anterior supernumerary cells (next to the border with the segmental) were sometimes divided.

These result suggest that

(i)The M blast cells can undergo some cell division without any signal from the environment (outside their own bandlet); however, the formation of somites (resulting from more cell divisions and organisation of those cells) may require signalling from the neighbouring lineages.

(ii) The macromeres may have a role to play in the organising of the bandlet, so that the cells organise themselves into a bandlet rather than a pole of cells. This role could be through signalling, or as a support for morphogenesis.

(iii) Even later, no cell death is observed in the bandlets in culture, until the whole culture degenerates (some stayed alive in culture for 72h; however, cultures degenerated at any stage). No difference between anterior and posterior produced blast cells was observed. In the control embryos, the anterior population of blast cells (segmental) is separated from the posterior (supernumerary) population by 1,2, or 3 elongated cells, morphologically very different. No such variation was observed in culture, and this could be due to the lower number of cells produced (i.e. supernumerary cells never being produced) as well as to the absence of

signal. The experiment did not allow to distinguish between the two hypotheses.

By isolating a cell from its natural environment, it is not only the chemical environment and molecular signals that we remove, but also the physical forces exerted on the cell. During the culture of the AB and CD cells, Symes & Weisblat (1992) have shown that mechanical constraints play a role in the normal pattern of cell division In my cultures, many constraints were removed: absence of the bulk of the embryo meant that the bandlets do not "crawl" on the same type of surface, and they do not have as much space to do so. By removing the other bandlets and therefore any possibility of forming a germinal band, the forces exerted on the bandlet are modified: the angles observed at the entry in the GB and in the GP suggest that there is some tension there; this tension could be responsible for shearing some of the cells (see Chapter II), but in the cultures, this tension does not exist any more.

On the basis of these results and interpretations, I have completed the model for germinal band (GB) size regulation, suggested in the previous chapter :

1. the blast cells born from the teloblast acquire an identity at birth equivalent to positional information for which portion of the body axis they belong to. At the same time they acquire a receptor to a signal for continuous cell division. The very first born cells hardly require any of the signal to go on dividing (sensitive receptor), while the more posterior cells require more signal to keep on dividing (relatively insensitive receptor).

2. At the border, all the cells are born with a very similar sensitivity to the signal, but only the more anterior ones make contact with the adjacent bandlets, and acquire enough of the proper signal to divide. More posteriorly, they may be able to divide once or twice only.

3. The observation that some cells die at the border between supernumerary and segmental cells may be the result of the more anterior cells getting organised into a tissue that still follows morphogenetic movements, and a posterior bandlet that cannot elongate anymore (the cells in it do not divide anymore and the teloblast has stopped dividing). The shearing of the cell happens a long time after the teloblast has stopped dividing, and after the segmental and supernumerary cells express their differences of division.

Conclusion

In summary, I suggest that supernumerary and segmental blast cells are different at birth with regard to cell division, with the consequence of this leading to different fates. The difference between them can only be brought about by an "encouraging" signal for the segmental cells to divide. I postulate that the signal comes from the other bandlets, and that it is the combination of cell program and signalling that eventually regulates the size of the germinal band. I also postulate that the macromeres might have a role to play in the organisation of the cells in an antero-posterior bandlet.

Chapter IV: Looking for *hairy (h)*, a segmentation gene, in the leech

Introduction

As defined earlier, the regulation of the number of segments in the leech involves setting a boundary at the posterior limit of the segmented body, after the 32nd segment. Are there any genes known to be involved in that sort of patterning? Looking at different systems can help understanding what kind of molecules are involved.

An obvious species to compare the leech with is *Drosophila*: annelids and arthropods are closely related (Kristan, et al., 1993), and both phyla are characterised by their segmental body plan. Much is known about the way *Drosophila* sets up segmentation (see Chapter I for summary of *Drosophila* development and gene expression), using a hierarchy of segmentation genes (Akam, 1987; Ingham, 1988). Many of the genes involved in segmentation in *Drosophila* are expressed at the critical time of segmentation in related arthropods and annelids, some of which set up segmentation very differently. This suggests that some of the segmentation genes might have been present before the arthropods and the annelids diverged. However, the setting up of segments by a gene hierarchy, as in *Drosophila*, might be only one of the possible adaptations.

Among the genes involved in setting up the segments, pair-rule genes are the first to show overt periodicity, appearing in alternate segments. The number of pair-rule stripes is therefore directly related to the final number of segments in the embryo. It was suggested that the prime function of pair rule genes is to "locate boundaries that delimit fields or gradients of positional information"(Lawrence, 1987). Thus pair-rule genes are good candidates for genes that have a function in locating the boundary between segmental and supernumerary tissue.

Among the pair-rule genes, *hairy (h)* is the earliest expressed, and regulates other pair-rule genes as well as segment polarity genes. *h* is therefore a very good candidate for being involved in the regulation of the number of segments in the leech. In this study, I have been looking for evidence of the presence of the *h* gene in the embryonic leech.

Background to the hairy (h) gene in development

The hairy gene is a pair-rule gene in Drosophila (segmentation gene)

During the setting up of segments in *Drosophila*, the pair-rule genes are expressed at the time when the syncitial blastoderm starts becoming cellular. In the segmentation genes hierarchy, they are downstream of the gap genes, and upstream of the segment polarity genes.

There are at least 8 pair-rule genes (Nüsslein-Volhard & Wieschaus, 1980), among which *hairy (h), runt (rnt), even-skipped (eve), fushi-tarazu (ftz),* and *paired (prd)* have been molecularly characterised. They are all expressed as 7 or 8 transient stripes during cellularisation of the blastoderm. The *h* mRNA is initially expressed uniformly through the embryo, and is one of the first pair-rule genes to express a restricted pattern of expression, in the form of seven stripes, all appearing more or less at the same time (Hooper et al., 1989). The protein itself only ever appears as a pattern of repeated stripes. The first stripes to appear are 1, 2, 3 and 7. Stripes 4 and 6 are fused to 3 and 7 respectively when first detectable. Once mature, the stripes are stable during the blastoderm stage, and decay at the onset of gastrulation. The *h* stripes do not appear in any antero-posterior gradient as do the *en* stripes (Hooper et al., 1989; Kornberg, et al., 1985; Patel, et al., 1989)

The protein is also found in an antero-dorsal patch, in the proctodaeum and in the nervous system (Hooper et al., 1989), but the site

(outside the segmented domain) and time of expression (after segmentation) is incompatible with any pair-rule function in those sites.

Since the expression of the pair-rule genes is the first indicator of a segmented structure, with the number of stripes relating to the number of segments, pair-rule genes may have a role in the regulation of the number of segments. In *Drosophila,* some of the stripes of another pair-rule gene,*eve*, are regulated by separate enhancers (Goto, et al., 1989; Harding, et al., 1989), and the pattern of expression of the segment polarity gene *en* is under the control of separate enhancers at different times of development (DiNardo, et al., 1988). The number of stripes in the descendants of the segmented ancestor could be related to the different enhancers they inherited, acquired and/or modified.

The pair-rule genes are not only required in the hierarchy of segmentation genes for the correct expression of downstream genes, they are also involved in germ band extension. After the onset of gastrulation, during germ band extension, cell intercalation takes place along the antero-posterior axis. It has been shown that segmentation genes, and more particularly pair-rule genes, affect active cell intercalation (Irvine and Wieschaus, 1994). Since cell intercalation in other systems has been shown to rely on differences in cell adhesiveness, it has been postulated that pair-rule genes establish stripes of cells that differ in adhesiveness (Irvine & Wieschaus, 1994).

The expression of pair-rule genes in other invertebrates

The understanding of the role of segmentation genes in pattern formation requires that systems other than *Drosophila*, closely related and less closely related, are studied in order to distinguish between the particular and the general. Recently, with the cloning of a range of pair-rule genes in a variety of arthropods (Patel, et al., 1992; Patel, et al., 1994; Sommer and Tautz, 1993; Sommer and Tautz, 1991; Sommer, et al., 1992), it

has been possible to evaluate the evolutionary relevance of the pair-rule genes, i.e. the role that these genes might have played in evolution for generating a variety of body plans. We can now address the questions of (i) whether these genes have a pair-rule function in these different organisms, and (ii), if so, whether this function can account for the regulation or change of segment number.

So far, in the species where segmentation genes have been studied, the expression that resembles the most the expression pattern in *Drosophila* is found in other long germ band insects. In the less closely related intermediate germ-band insects (e.g. the beetles), *eve* and *h* were found to be expressed in stripes, appearing as the blastoderm grows, and preceeding the expression of the *en* stripes in a way consistent with their putative role as pair-rule genes (Patel, 1994). On the other hand, no evidence of pair-rule pre-pattern, i.e. pattern of double segment periodicity, was found in *Schistocerca* (Patel et al., 1992), a short germ band insect more distantly related to *Drosophila*. The *eve* pattern in this insect was not expressed in stripes at the moment of segment formation.

However, the majority of insects are not long germ band insects, be they closely or distantly related to *Drosophila* (Anderson, 1973): *Schistocerca* is a short germ band insect, and the beetles intermediate-band insects. Short and intermediate germband insects differ from *Drosophila* by the fact that they are cellularised at the time their body plan is being established. This makes it impossible for genes such as the maternal genes and the gap genes to be acting in a similar way: in *Drosophila*, the product of these genes can diffuse through the syncitium, and form a gradient, with different nuclei responding differently depending on their position along the axes. In the other insects (short and intermediate-band), gradients of such molecules cannot be formed due to the presence of cell membranes(Patel, 1994). Short germ band insects also set up their tissue differently, with the presence of a

growth zone at the posterior end of the embryo. In a way, it could be argued that the short germ-band and intermediate germ band insects develop more like the leech than *Drosophila*, considering that they form their segmental tissue by division of a growth zone on the same principle as the teloblasts of the leeches. However, there is no evolutionary evidence to suggest that the mechanism in both phyla was inherited from a common ancestor developing through a growth zone (Anderson, 1973).

If segmentation is set up differently at the cellular level, it can be argued that it might also be different at the molecular level. However, the different types of insect development (long, intermediate and short germ-band) do not reflect the true phylogeny of the class: these different types of development are scattered among the insect classes, suggesting a polyphyletic origin. The most recent studies put the emphasis on differences at the oogenesis stage: the meroistic/panoistic dichotomy among the insects, which seems to correlate better with their evolution (French, 1990). In the meroistic ovary, such as in *Drosophila* the oocyte is associated with nurse cell via cell junctions, allowing the diffusion of large molecules, such as maternal RNA. In the panoistic insect, there is no such contact. The break of symmetry of the new egg has to be imparted differently, and the hierarchy of segmentation has to start differently: there has been so far no reports of a homologous *bicoid* gene found in any other organism than *Drosophila*.

How the segmentation pattern has evolved is still not very clear, and we cannot, on the sole basis of the comparison of patterns of expression in the arthropods, predict whether a pair-rule gene pre-pattern exists in the leech. There is some evidence to suggest that some of these pair-rule genes may be involved in the regulation of the number of segments: in *Drosophila*, pair-rule genes are expressed in 7 stripes. In the beetles, they are expressed

in 8 (Patel et al., 1994), and this correlates to an increase in the number of abdominal segments from 8 in *Drosophila* to 10 in the beetles. In *Drosophila,* the expression of a specific gene is governed by a distinct subset of *cis*-acting elements in each stripe(DiNardo et al., 1988; Harding et al., 1989). The "tinkering" of the enhancer is a powerful tool for generating diversity even among closly related organisms: an increase in the sensitivity of the enhancer site to the transcription factor or vice-versa could increase the number of stripes; however, since there has been no such mutants found in the extensive *Drosophila* mutant screen, it is possible that such a difference could not happen in one step. This could, however, be a means of regulating the number of stripes of expression (and segments) in the various related organisms that have inherited pair-rule patterning, and in the leech if it also descends from a pair-rule patterned ancestor.

Segmentation genes in the leech

As was described in the earlier chapters, the leech sets up segmentation anteroposteriorly (see Chapter I for a description of the developmental stages): as the blast cells mature in a temporal gradient, the first born blast cells divide and produce segments first. This way of developing is very common. It can be found in a variety of systems, in different organisms with stem cells, such as arthropods (e.g. the crustaceans), that develop through a posterior growth zone (Anderson, 1973).

In the leech, cell lineage studies have been carried out to understand the relationship between blast cells and segments (Weisblat & Shankland, 1985). The results showed that one blast cell from the M, the O and the P lineage and two blast cells from the N and the Q lineage would develop to form one hemisegmental complement. The descendents of the blast cells are not however restricted to a compartment as defined by Garcia-Bellido: "developmental compartments are discrete areas of tissue produced

exclusively by all the descendents of a small group of founder cells that contribute only to this structure"(Garcia-Bellido, 1975). For example, descendants of the third born blast of the M teloblast will populate part of the segments 2, 3 and 4 (see in Chapter I, Figure I.5). It is not known whether some information and signalling is required for the descendents of the blast cells to reach the proper segment: cells from different lineages but in the same segmental register do not come together until late, at which point their segment identity is at least partly determined (Lans et al., 1993). However, considering that the pattern of all the blast cells is the same (in terms of which descendents will populate which segment) for all the segments, it can be suggested that this information is encoded in the cells at birth or soon after birth. However, there is no evidence that this is true.

On the other hand, the number of segments itself cannot be cell-autonomous as the teloblasts undergo a variable number of cell divisions to give rise to the blast cells, the founders of the segments. Therefore, we could argue that at least the last segment needs its posterior limit to be fixed.

The segmentation genes so far reported in the leech are the genes involved in the differentiation of the segments in *Drosophila* (homeotic or segment identity genes) (Aisemberg and Macagno, 1994; Master et al., 1994; Nardelli-Haefliger and Shankland, 1992; Nardelli-Haefliger and Shankland, 1993; Wysoka-Diller, et al., 1989) and two segment polarity genes, *en* (Lans et al., 1993) and *wnt* (Kostriken & Weisblat, 1992). The *lox2* gene, a *Ubx* homologue, is expressed in a subset of segments (Nardelli-Haefliger et al., 1994), a typical segment identity pattern. The *en* gene is expressed in the same subset of cells in all the segments, very much like segment polarity genes in *Drosophila*, and even in the same section of the segment, i.e. in a posterior patch, like its homologue. However, because of their rather late expression, these genes (the segment polarity genes and the segment

identity genes) are probably involved in the patterning within the segments rather than the setting up of the segment *per se* , if involved at all!

Of the genes expressed earlier in the *Drosophila* hierarchy, only a gap gene, hunchback, has been reported in the leech so far, but no expression pattern is available yet (Savage and Shankland, 1994).

Molecular aspects

The hairy gene is part of a basic Helix-Loop-Helix family

The *h* gene is a transcription factor, and part of a basic Helix-Loop-Helix (bHLH) family of genes. This family comprises *h* homologues from various species, *Drosophila virilis* (Rushlow, et al., 1989), *Musca domestica* (Sommer & Tautz, 1991), *Tribolium castaneum* (Sommer & Tautz, 1993), and other bHLH genes such as the enhancer-of-split gene, found in *Drosophila* (Delidakis and Artavanis-Tsakonas, 1992) , in rat and mouse (HES_1, HES_2 and HES_3) (Ishibashi, et al., 1993; Sasai, et al., 1992) and in other vertebrate species (Domingos Henrique, unpublished), (see Figure IV.1). These vertebrate homologues exhibit similarities to both *h* and *enhancer-of-split* (see Figure IV. 2). These genes are all expressed during development, in the developing nervous system. There are other related genes, such as *deadpan,* another neurogenic gene (Bier, et al., 1992)., and the *h* gene also shows homology to N-*myc* (Rushlow et al., 1989).

All these genes share sequence similarities in the bHLH domain and in the WRPW domain at the C-terminal end of the protein (Wainwright and Ish-Horowicz, 1992). The fact that the *h* gene has so many homologues or closely related genes that are known, has allowed us to design efficient strategies of cloning by homology .

```
.          251                                                  300
 Dmhairr   ...QQGAAAP YLFG.IQQTA SGYFLPN..G MQVIPTKLPN GSIALVLPQS
Dvhairyj   SSNNTNTTAP YLFGQIQQNA NGYFLPN..G MQVIPTKLPN GSIALVLPQS
 Tchairy   .......... ......EVAP NNIILGNGTG VQLVPIRLAN GDIALVLP..
 Rnhes1a   GAAPPPGSAP CKLGSQAGEA AKVFGGFQVV PAPDGQFAFL

           301                                                  350
 Dmhairr   LPQQQQQQLL ...QHQQQQQ QLAVAAAAAA AAAA...... QQQPMLVSMP
Dvhairyj   LPQQQQQQLL QQHQHHQQQQ QLAAAAAAAA AAAAAVAQQH QQSPLLVAMP
 Tchairy   .......... .......... .......... ......TQGA SPLPLLVPIA
 Rnhes1a   IPNGAFAHSGP VIPVYTSNSG TSVGPNSNSG

           351                                                  400
 Dmhairr   QRTAS1GSAS SHSSAGYESA PGSSSS.CSY APSSPANSSY EPMDIKPSVI
Dvhairyj   QRTASTGSAS SHSSAGYESA PSSSSSRGSY APPSPANSAY EPMDVKPSVI
 Tchairy   .......... .......... ....SSASNY SPSQSP.... EPESVR....
 Rnhes1a

           401                                       437
 Dmhairr   ....QRVP... MEQQPLSLVI KKQIK.EEEQPWR PW*
Dvhairyj   ....QRVPHMH LEQQPLSLVI KKQIKVEEEQPWR PW*
 Tchairy   ..........  ....PLSLVV RRREEPTEEKPWR PVVETVM
 Rnhes1a                          TSVGPNTADSMWR PWR N*
```

Figure IV. 1 Comparison of the protein sequence of *h* in different species. (Dmhairr: *Drosophila melanogaster;* Dvhairyj: *Drosophila virilis;* Tchairy: *Tribolium castaneum;* Rnhes1a: *Ratus norvegicus*). The basic region is underlined, the helices are in bold and the loop region in italics. Note how the different species are highly conserved only in the bHLH domain and the WRPW domain. The size of the peptide between the bHLH domain and the WRPW fragment varies even within insects (*D. melanogaster, D. virilis* and *T. castaneum).*

```
          1                                                  50
 Dmhairr  .......... .......... .......... .......... ........MV
Dvhairyj  .......... .......... .......... .......... ........MV
 Tchairy  .......... .......... .......... .......... ..........
 Rnhes1a                    RGRKQKNIFF CVKDSKNKIL WGLRRKKKRKWGL

          51                                                 100
 Dmhairr  TGVTAANMTN VLGTAVVPAQ L......KET PLKSDRRSNK PIMEKRRRAR
Dvhairyj  TGITTTMSPN VLGTAVVSTQ QQQQQQHKEA PIKSDRRSNK PIMEKRRRAR
 Tchairy  .......... .......... .......... .......SNK PIMEKRRRAR
 Rnhes1a  RRKKKRKMPADIMEKNSSSPVAATPASVNTTDDKPK  TASEHRKSSK PIMEKRRRAR

          101                                                150
 Dmhairr  INNCLNELKT LILDATKKDP ARHSKLEKAD ILEKTVKHLQ ELQRQQAAMQ
Dvhairyj  INNCLNELKT LILDATKKDP ARHSKLEKAD ILEKTVKHLQ ELQRQQAAMQ
 Tchairy  INNSLNELKT LILDAMKKDP ARHSKLEKAD ILEMTVKHLQ NLQRQQAAMW
 Rnhes1a  INESLSQLKT LILDALKKDS SRHSKLEKAD ILEMTVKHLR NLQRAQMTAA

          151                                                200
 Dmhairr  QAADPKIVNK FKAGFADCVN EVSRFPGI.E PA...QRRRL LQHLSNCING
Dvhairyj  QAADPKIINK FKAGFADCAN EVSRFPGL.D ST...QRRRL LQHLSNCING
 Tchairy  QPTDPSVVSK FRAGFSECAS EVGRFPGLDE PV...VKRRL LQHLASCLNQ
 Rnhes1a  LSTDPSVLGK YRAGFSECMN EVTRFLSTCE GVNTEVRTRL LGHLANCMTQ

          201                                                250
 Dmhairr  VKTELHQQQR QQ...QQQSI HAQ.MLPSPP SSPEQDS... ..........
Dvhairyj  VKTELHHQQR QQALAQAQSL HAQVVLPSPP SSPEQEPSVT PVAASGNNNS
 Tchairy  GQKE..... .........P QVQVIVP... .......... ..........
 Rnhes1a  INAMTYPGQA HPAL QAPPPPPPSG PGGPQHAPFA PPPPLVPIPG
```

Figure IV. 2 Alignment of sequences of the seven E(spl) proteins and the hairy preotein (h). To highlight similarities, bold upercase letters indicate residues that are identical in all E(spl) proteins (hairy is shown only for comparison and is disregarded for the above highlighting). Also, for comparison, is included the bHLH region of scute (l'sc); there is no homology with other regions of l'sc (Delidakis and Artavanis-Tsakonas, 1992).


---BASIC---
m5 mapqsnnsttFVSKTQhYl-KVkKPLLERqRR- (31)
m8 .....MeYtTKTQiYq-KVkKPMLERqRR- (23)
m7 .....matkyEMSKTYQYR-KVMKPLLERKRR- (26)
mA .....mvlemEMSKTYQYR-KVMKPMLERKRR- (26)
mB ...msslqMsEMSKTYQYR-KVMKPMLERKRR- (28)
m3 .....mVmEMSKTYQYR-KVMKPLLERKRR- (24)
mC ...mavqgqrFMTKTQhYR-KVtKPLLERKRR- (28)
h mvtgvtaanmtnvlgtavvpaqlketplksdR-RsnKPIMEKRRR- (44)
lsc svaR-R...nArERnRv-
---HELIX I--- ---LOOP---
m5 ARMNKCLDtLKtLVAE-fq...GDdAIlRMD- (58)
m8 ARMNKCLDnLKtLVAE-lr...GDdGIlRMD- (50)
m7 ARINKCLDELKDLMAE-CVaQcGD.A..KFE- (53)
mA ARINKCLDELKDIMVE-CLtQEGEH.ITRLE- (55)
mB ARINKCLDELKDLMVa-tLesEGEH.VTRLE- (57)
m3 ARINKCLDDLKDLMVE-CLqQEGEH.VTRLE- (53)
mC ARMNlyLDELKDLIVD-tMdaqGEq.VSKLE- (57)
h ARINnCLnELKtLILD-atkkDparh.SKLE- (73)
lsc kqVNngFvnLRqhLpq-tVvnslsngGrgssk..KLs-
--HELIX II--
m5 KAEMLEAaLvFMRKQ-vvkqQAp.....VSPLPm (86)
m8 KAEMLEsaViFMRqQ-KtpKkVaqeeq.....S.LPl (80)
m7 KADILEVTVqHLRKL-KesKkhvpan.....PE (80)
mA KADILELTVeHMKKL-RaQKQLrlssvtgg.VSPsaDpklsia (96)
mB KADILELTVtHLqKM-KQQRQhkrasgdes.LTPA (90)
m3 KADILELTVdHMRKL-KQrggLslqgvvagvgSPptststahv (95)
mC KADILELTVnYLKaQ-qQQRvAnpq.....SPpPDqvnl (90)
h KADILEkTVkHLqeL-qrQqaAmqqa.....aDpkiv (104)
lsc KVDtLrIaVeYIRgL-qamld...
m5 DSFKnGYMNAVsEISRVMAcTPAMSVDVGKTVMTHLG (123)
m8 DSFKnGYMNAVNEVSRVMASTPGMSVDLGKSVMTHLG (117)
m7 qSFRAGYIRAANEVSRALASLPrVdVaFGTTLMTHLG (117)
mA ESFRAGYVHAANEVSKtLAaVPGVSVDLGTqLMSHLG (133)
mB EGFRSGYIHAVNEVSRsLSqLPGMnVsLGTqLMTHLG (127)
m3 ESFRSGYVHAAdqITqVLLqTqq.TdEIGRkIMkfLS (131)
mC DkFRAGYtQAAyEVSHIFSTVPGLdLkFGThLMkqLG (127)
h nkFKAGFAdcVNEVSRF....PGIepaqrrrLLqHLs (137)
m5 veFQrMlQadqvqtsvttstpr.....pLSPASSGYhSDnEdsqsaaspkpveet
m8 rvykNLQQfheaqsaadfiqnsmdcssmdka.....pLSPASSGYhSDcDspppt pqpmqqp..
m7 mRLNQLeQpmeqpqavntplsivcqsssssstyssasscssISPVSSGYaSDnEsllqisspqq....
mA hRLNyLQvvvpslpigvplqapvedqamvtpppsecdtlesgrcspapseasstsqp.....
mB qRLNQIQpaekevlpvtaplsvhianrdavsvpispissvaqspnsntsstshsllttidvtkmeddsedeen
m3 tRLieLQtqllqqqqqqqqhqqqqipqssgrlafpllggygpaaaaaaisyssfltskdelidvtsvdgnal setasvssqesqasep
mC hqLkdMkQeeeiidmaeepvnladqkrskspreedihhgee.....
h ncINgVktelhqqqrqqqqqsihaqmlpsppsspeqdsqqgaaapylfgiqqtasgyflpngmqviptklpn gsialvlpqslpqqqqqqllqhqqqqqqlavaaaaaaaaaaqqqpmlvsmpqrtastgsasshssagvesap qsssscsvappspanssyepmdikpsviqrvpmeqqplslvikkqikeeeq
m5 MWRPW (178)
m8 LWRPW (179)
m7 VWRPW (186)
mA MWRPW (195)
mB VWRPW (206)
m3 VWRPW (224)
mC VWRPW (173)
h pWRPW (337)

Figure IV.2

The techniques involved in cloning the genes controlling segmentation

Many genes have been isolated in *Drosophila* by exploiting the ease of genetic manipulation in this organisms, and also the possibility of generating mutants and testing complementation. The approach in other organisms, has been to isolate the homologues (based on their sequence) of the segmentation genes, by library screening or by PCR. Once homologues are isolated, their function is much more difficult to detect than in *Drosophila* and, in the leech, can only be done by comparison. The first thing is to see when and where it is expressed in leech segmentation in relation to the other genes. The second, and more conclusive, test is the complementation test in the *Drosophila* mutant, using the newly found gene from a different species. Positive complementation (rescue) of the mutant is a sign of homology, and suggests that the gene might have the same function in its original environment/background (Krauss, et al., 1993); but it is not enough to prove that the molecule has the same function in the organism where it was isolated.

In this chapter I describe how I have looked for evidence of the presence of the *h* gene in the leech, using a combination of Southern blot and PCR techniques. My results show that the *h* gene may not have a closely related homologue in the leech.

Methods

Southern Blot

DNA extraction from adult leeches:

The adults were weighed by resting them on tissue paper on the balance. The yield was different depending on the species: 16 adult *Theromyzon* weighed about 4 g (yield 40 mg DNA, i.e. 10mg DNA per g of tissue or 2.5 mg of DNA per adult); 100 adult *Helobdella triserialis* weighed

about 1.5g (yield 40 mg DNA, i.e. 26.67 mg of DNA per g of tissue, or 0.5 mg DNA per adult); 20 adult *Glossiphoniia complinata* weighed about 1.8g (yield 40 mg DNA, i.e. 22.22 mg of DNA per g of tissue or 2mg of DNA per adult); 4 adult *Hirudo medicinalis* weighed about 2g (yield 200 mg DNA, i.e. 100mg of DNA per g of tissue or 500mg per adult). They were ground in liquid nitrogen, with a mortar and pestle, preferably on dry ice, to consistency of a fine powder.

10 ml of grinding buffer (0.1 M Tris, 0.05 M Na_2EDTA, 0.2 M Na, 1% SDS) was added per gram of tissue. Proteinase K was added to 100 mg per ml. The mixture was left at 60/65°C overnight.

The mixture was extracted with phenol:chloroform:isoamylalcohol 25:24:1, adding the same volume. After mixing of the two phases, it was centrifuged at 5000g for 20 min, and the supernatant poured into a new tube.

The supernatant was re-extracted until it became clear, usually 3 times. Some pigments always stayed attached to the DNA but they did not seem to affect the quality of the DNA.

The extract was then ethanol precipitated with sodium acetate (1/10th volume of sodium acetate, and 2 volumes of ice cold ethanol). At this point, the DNA was sometimes spooled out, or the tube is mixed delicately, and kept frozen at -20°C until further use.

After thawing and centrifugation (5 minutes at 5000g), the DNA was resuspended in 100µl of TE.

Estimation of the amount of DNA

When possible, the DNA concentration was estimated by reading the optical density (O.D.) at 260 nm of the diluted DNA solution, and using the formula:

$$1u\ O.D._{260} = 50\mu g/ml\ DNA$$

Because the concentration of DNA does not have to be too precise, if the total amount was too low for the O.D. method, DNA concentration was estimated by loading a small (diluted) amount of DNA on a gel, stained with ethidium bromide. The concentration of the DNA was estimated by comparing (by eye) the intensity of the DNA sample to the intensity of a DNA in known concentration run in a different lane of the same gel: I used HindIII cut Lambda DNA as a marker, at the concentration of 250ng/µl, and a total loading of 500ng. The relative amount of DNA contained in each of the DNA fragments was easily established by proportional relationship. This allowed a sufficient estimate of the order of magnitude of the concentration of the DNA extracted (compared to the O.D. estimate).

Restriction Digest

DNA was digested with the EcoRI enzyme. A typical restriction digest was: 100µl DNA (20µg), 10µl EcoRI enzyme (100 units), 10µl buffer H (Boehringer) and 70µl ddH_2O, mixed together, and incubated overnight at 37°C. The resulting digest was checked on a gel by loading 10 µl of a typical 200µl digest. If the smear was long and regular, and no very large molecular weight band was observed, I assumed the DNA was well cut. If the digest was not extensive, more enzyme was added and the reaction prolonged for 1h to overnight, then checked again.

Agarose gel

The DNA from the different species was loaded in equivalent amounts (4µg) on a 1% agarose gel, running at about 100mV and 100mA. Before blotting, a photograph was taken with a ruler so that the size of the fragment appearing in the Southern blot could be recognised later. I used the HindIII cut Lambda DNA as a DNA fragment length marker (500ng).

Making the probe

The probe used in the *hairy* Southern blot experiment was given by Mark Wainwright in David Ish-Horowicz's lab in the form of a plasmid . The THΔ1 probe was a 3'end fragment of a *Drosophila hairy* genomic clone (refer to Figure IV.1) that did not contain the insert, but did contain the WRPW motif, and of total length 1.3kb. The probe has not been characterised further but it is known to recognise 2 fragments in *Drosophila* of size 4kb and 2.5kb (see Results for detail).

The probe was generated using the Boehringer DNA DIG-labelling system. First, the insert was cut from the plasmid by doing a double digest, using EcoRI and HindIII. A typical digest was: 50µl plasmid DNA (obtained by the mini-prep of one 1.5ml culture, estimated to be about 3µg), 1µl EcoRI (50 units), 3µl Hind III (32µl), 7µl reaction buffer 2 (New England Biolab), and 9µl of water to 70µl. The total digest was run on a 1% agarose gel and the appropriate band was recuperated by cutting the 1.3kb band and extracting it using the Costar gel-extraction kit. The amount of DNA extracted was estimated by running a small amount on a gel, and a typical labelling reaction used 15µl DNA (approximately 1ng/µl) and was done using the Boehringer DNA random-primed labelling kit, and resuspended in a total volume of 25µl. The total amount of the probe was estimated by colour reaction on dot blots. I usually obtained a yield of 50ng per reaction. This was enough for one Southern blot.

Southern blot hybridisation conditions

The procedure followed was based on Boehringer Mannheim GmbH, Biochemica " The DIG system user's guide for filter hybridisation", 1993. The 1% agarose gel was blotted onto a positively charged nylon membrane (Boehringer), and UV cross-linked. The conditions for prehybridisation, hybridisation and washing followed the manufacturer's recommendations, with the adaptations described in the Results section. The probe was used at

the concentration of 15ng/ml. The detection was done either by colour reaction (using NBT and X-Phosphate, Boehringer), or by chemoluminescence (using Lumigen, Boehringer). The membrane was then stripped using N-N-Dimethylformamide (only when the colour reaction was used) and 0.2N sodium hydroxide, 0.1% SDS (probe stripping solution) to remove the probe (for both the colourimetric and the chemoluminescent methods). Different conditions were used for attaining the optimal hybridisation.

High stringency conditions:

Hybridisation	Washes	
68°C in 5x SSC	2x at R.T., 0.1x SSC	2x 68°C, 0.1xSSC

Medium stringency conditions:

Hybridisation	Washes	
68°C in 5x SSC	2x at R.T, **2xSSC**	2x **45°C, 2xSSC**

Low stringency conditions:

Hybridisation	Washes	
65°C in 5x SSC	2x at R.T, 2xSSC	2x 45°C, 2xSSC

PCR

Preparation of the DNA from the library

I used a cDNA library from M.Shankland's lab (Shankland et al., 1991), made from stage 9-11 *H.robusta* embryos. The isolation of DNA from bacteriophage λ was done by rapid, small-scale isolation as described in (Sambrook, et al., 1989), and slightly adapted:

Plating bacteria were first made, by inoculating 1 colony of XL1 blue into 5 ml of LB containing 0.4% maltose, and grown overnight, shaking. 1ml of the culture was added to 50 ml of prewarmed LB containing 0.4% maltose, in a 1l flask. The cells were incubated with vigorous shaking for about 4h until the OD_{600} reached 0.5 (i.e. $2.5x10^8$ cells/ml). The culture was

cooled on ice, centrifuged at 3000rpm for 10 minutes, and the bacterial pellets resuspended by vortexing in 15ml of ice-cold 10mM $MgCl_2$. The cells were stored at 4°C.

A **liquid culture** was produced by mixing 0.5ml of plating cells with 1-5 µl of library lysate (pure or diluted in SM), at a total number of 10^7 pfu, although higher and lower titers (up to a factor of 5) still gave lysates. The phage was adsorbed by leaving the cells and phage for 15 minutes at room temperature. 5 ml of LB was added with 5mM of $CaCl_2$ and the culture incubated at 37°C with vigo rous shaking. The tubes were monitored for lysis, which was usually visible after 3 to 5h. A few drops of chloroform were added, and the culture left shaking for a few more minutes. The lysate was centrifuged at 3000rpm for 10 minutes, the supernatant removed to a new tube, 1 drop of chloroform added and kept at 4°C. The stock was titred to check it was higher than $2x10^{10}$pfu/ml.

For the **Lambda DNA preparation**, to a 5ml λ liquid culture, RNAse A and DNAse I (crude: Sigma) were added to the supernatant to a final concentration of 1 µg/ml each, and the reaction incubated at 37°C for 30 min. An equal volume of cold 20% polyethylene glycol (PEG) / 2M sodium chloride in storage buffer (SM) was added to precipitate phage particles. The reaction was incubated for 1h on ice. The phage were recovered by centrifugation at 10 000 g for 20 min at 4°C. The supernatant was discarded and 0.5ml SM was added to the pellet, resuspended by vortexing.The solution was centrifuged at 8 000g for 2 min at 4°C to remove the debris. The supernatant was transferred to a new tube with 5 µl 10% SDS and 5 µl 0.5M EDTA (pH8), incubated at 68°C for 15 min. The DNA was extracted once with phenol-chloroform-isoamylalcohol (25:24:1), and once with chloroform-isoamylalcohol (24:1). The final aqueous phase (supernatant) was poured into a 2ml microfuge tube. To this final aqueous phase I added: 1/10th volume of 3M sodium acetate (pH5.5) and 2x the final volume of

100% ice-cold ethanol. After precipitating the solution for minimum 2h at -20^{0}C, it was centrifuged 15 min at 14 000g, washed with 70% EtOH, dried and resuspend in 100 µl TE (pH8). The concentration was checked on a gel, and 10µl was used for restriction digest. If the DNA was reticent to enzymatic reaction, it was phenol extracted and ethanol precipitation was repeated.

The critical step was the precipitation and resuspension of the DNA. Other methods tried (e.g. Sambrook et al., 1989 or Amersham's own protocols for handling λgt11 phage) gave a very low yield or did not allow the pellet to resuspend. This problem was solved by precipitating the DNA using ethanol rather than isopropanol. This way, the yield was of 15 to 25 µg per 5ml lysate (i.e. starting with 10^7 pfu).

RNA extraction and first strand synthesis

Again, several methods were tried and compared. Probably because of the amount of yolk contained in the embryos at the stage I was working on (early stage 8 and juvenile), the micro-mRNA extraction kits (from Pharmacia), containing guanidium, did not give satisfaction, precipitating the proteins into a lump. I used a protocol for extracting whole RNA (Sambrook et al., 1989) ,with a further RNA purification from yolk proteins using Li Cl_2. One extraction used 1 batch of 3 cocoons (of *T. tessulatum*, the species with the largest available eggs) layed by the same mother, giving a total of approximately 100 embryos to a volume of 100µl. The total RNA was redissolved in 12µl and the totality used for the first strand synthesis as a template for reverse trancription.

The first strand synthesis was done using the Boehringer enzyme Superscript II. Different first strands were made by using different primers: (i) The downstream primer used in the PCR (such as W, see PCR section, and Figure IV. 3), permitted specific transcription, and was followed by the use of nested primers in the PCR (50pmol per 20µl reaction); (ii) A polydT

(A)

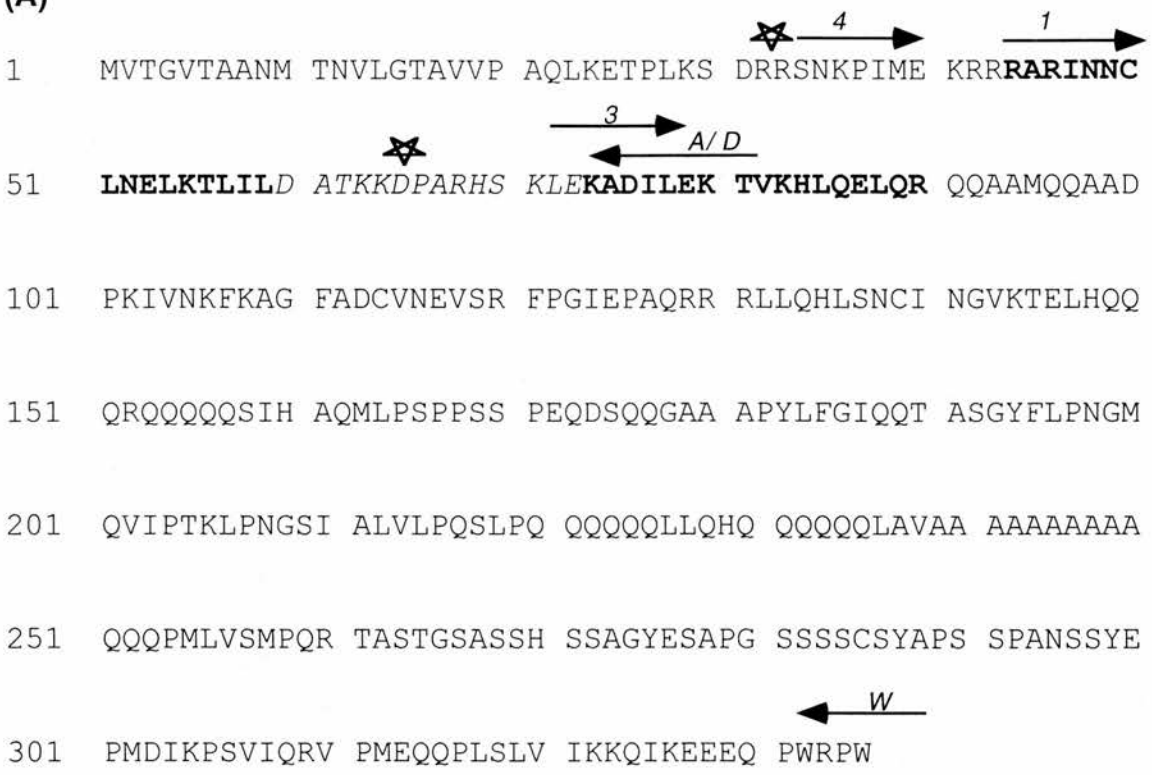

Figure IV.3 The pimers used in the PCR reactions for *h* in the leech. **(A)***D. melanogaster* sequence of hairy with localisation of the primers used. The HLH domain is highlighted with the helices in bold, the loop in italics. The primers 1, 3, 4, A and W are represented by arrows pointing in the direction of polymerisation. Position of introns (in *Drosophila*) are marked by a star. The sequence beween the end of the helix and the conserved WRPW motif at the C-terminal varies in sequence and in size even among closely related species. **(B)** The sequence of degenerate primers. Top line is the nucleotide sequence, bottom line is the amino acid translation where appropriate

(B)

Primer A

AC	IGT	CTT	CTC	NAG	NAT	STC	NGC	YTT
V	T	K	E	L	I/M	D/E	A	K

Primer B

TC	NAG	CAT	CTC	IGC	CTT	GTC	CAT
(E)	L	M	E	A	K	D	M

Primer W

CGT	CTA	CTYA	CCA	IGG	ICK	CCA
			W	P	R	W

Primer 1

CGI	GCI	CGI	ATN	AAC	AAN	TGY	YT
R	A	R	I/M	N	K/N	C	L

Primer 3

GTC	GAC	AAG	GCI	GAY	ATY	YTI	GA(A/G)
L	E	K	A	D	I	L	E

Primer 4

AAC	AAR	CCI	ATH	ATG	GAR	AAR
N	K	P	I	M	E	K

Primer D

TTT	CTC	IAR	IAT	RTC	IGC	YTT
K	E	L	I	D	A	K

SYMBOLS FOR MIXED MERS

B = (C,G,T)
D = (A,G,T)
H = (A,C,T)
K = (G,T)
M = (A,C)
N = (A,C,G,T)
R = (A,G)
S = (C,G)
V = (A,C,G)
Y = (C,T)
W = (A,T)

primer (200ng per 20 µl reaction) was used for reverse transcribing mRNA non specifically. This primer would allow longer cDNA to be generated with the use of the Superscript II. The primer I used for this was the primer provided in the First Strand cDNA kit (Pharmacia), and contained a NotI site, so that in the PCR a primer specific to that site could be used instead of the polydT, which was feared too unspecific. (iii) A mixture of hexanucleotides (200ng per 20 µl reaction) was used for random priming.

Subsequently, I used 5µl of the reaction, straight into the PCR reaction.

PCR conditions

The cDNAs from the library or reverse transcription of mRNA were positively controlled for amplification. For this I used (i) the set of primers 879 (CTCGAG AAA TTC AAT CGC TAC CTC ACC) and 880 (GAATTC CTG GAC CTC TTT TTT CTC CTT), degenerate *engrailed* primers, amplifying a 130 bp fragment in leech genomic DNA and cDNA or, (ii) on the *H. robusta* cDNA library only, a set of primers *twist* and T3, which amplify a 300bp fragment by recognising a site in the *twist* gene and the T3 site in the plasmid (used for isolating *twist* from the library, J. Soto and D. Weisblat, personal communication). The basic conditions are described here. The specific conditions used in the PCR are described in the Result section. See Figure IV.3 for primer sequence and localisation.

The PCR mixture was 4.5 µl Taq 10x buffer, 1µl dNTP 10mM, 1µl of each primer at 0.5 µg/µl, 5 µl DNA (variable concentration), $MgCl_2$ concentration was adjusted to 1.5 mM, and water added to 45µl total. For PCR at low stringency, the concentration of $MgCl_2$ was 3mM. The mixture containing theTaq enzyme (0.5µl Buffer 10x, 1.5 units Taq, water to 5µl final) was added after the hot start. The final reaction volume was 50µl. The temperature conditions were: Hot start at 95°C for 5 minutes for complete denaturation and specific binding, and 75°C until the Taq mixture is added.

This was followed by 40 cycles of denaturing (92°C, 30"), annealing (50°C, 1') and polymerising (72°C, 30") with intermediate temperature steps between annealing and polymerising (55°C, 30"; 60°C, 15"; 65°C, 10") to allow for the Taq to start polymerising as soon as annealing had occured. A final step at 72°C for 5' was done to ensure all the new strands would be polymerised to completion.

The PCR was checked by running 5µl on an agarose gel.

Cloning

The PCR reaction was run on an agarose gel, and bands of the appropriate size were cut out and extracted, using the Quiagen ("Quiaex") kit, and resuspended in 20µl. Ligation was done using the pGem-T vector system from Promega. A typical reaction was: 1µl T4 DNA ligase buffer, pGem vector 1µl (50ng), PCR product 7µl, T4 DNA ligase 1µl. The reaction was incubated for 3h at 15°C, and stopped by incubating at 70°C for 10 minutes. Supercompetent cells DH5α (Inoue, et al., 1990) were transformed using 1µl of the ligation reaction. A control was run at the same time with no insert to test the quality of the plasmid and estimate the background. I used the blue-white colour reaction for selecting the clones with insert. But because of the small size of many of the inserts (<200bp), the recombinants were often masked as they developed white colonies. For this reason, a mixture of both colonies were tested by mini-prep (Magic mini from Promega) and double digest to excise the fragment from the plasmid. A typical digest was: 5µl DNA (from 50µl of the Magic mini, i.e. about 300 to 500 ng), 1.5µl buffer H (Boehringer), 0.1µl PstI (1 unit), 0.1µl SphI (0.5 units) and 8.3 µl ddH_2O, incubated at 37°C for 1h. The whole reaction was run on a 1% agarose gel. The clones containing inserts of the right size were selected for sequencing.

Sequencing: Sanger di-deoxy method

I used a variation of the Sequenase protocol, as follow. To 16 µl of the 50µl DNA obtained with the magic mini-prep (Promega) (approximately 2 µg), I added 4 µl NE (NaOH 1M, EDTA 1mM) and denatured the DNA by heating at 65°C for 5 min. The mixture was precipitated by transfering it to ice, and adding 2.5µl sodium acetate (3.5M) and 60 µl ethanol (cooled at 70°C). The tubes were put on dry ice for 15 min or at -20°C for 2h. The DNA was centrifuged at 5 000g for 10 minutes, and the pellet washed with 750µl 70% ethanol, then dried and resuspend in 7 µl water. From then on I followed the Sequenase protocol: annealing to the primer was done at 37°C, for 10 to 15' before transfering to ice for the labelling/polymerising steps. The primers were T7 sequencing primer (5' TAA TAC GAC TCA CTA TAG GG 3') or SP6 sequencing primer (5' GAT TTA GGT GAC ACT ATA G 3') at 0.1 to 1pM.

The sequencing gel was a 6% Long Ranger ready mixed polyacrylamide, polymerised with the addition of 40 µl TEMED and 400 µl 10% Ammonium Persulfate per 50 ml of polyacrylamide. The gel was run at 60W for 2 to 4h. It was then fixed in 10% acetic acid, 10% methanol for 10 minutes, blotted onto Whatman blotting paper, dried under vacuum at high temperarure (80°C) for 1 to 2h, and exposed to Agfa Curix X-ray film for 12 to 48h before developing.

Results

In this study, I have tried to find evidence for the *h* gene in the development of the leech. The approach was to use techniques of cloning by homology.

Southern Blot

The DNA was first extracted and its concentration estimated on a gel (see Figure IV.4). It was then cut using EcoRI, a rare cutter (6 cutter), which

Figure IV. 4 Estimation of the amount of DNA extracted from the different species of leech. 500 ng of λ/Hind III cut marker (L) were loaded in the first lane and in the last lane, as a reference, size markers (in kb) are to the left of the first lane ; the equivalent amount of each fragment is written to the right of the last lane in ng. *Helobdella triserialis (Ht)*: 400 ng DNA from 100 adults ; *Helobdella robusta(Hr)*: 200 ng DNA from 100 adults; *Glossiphonia complinata (G.c.)*: 400 ng DNA from 20 adults;*Theromyzon tessulatum (T.t)*: 400ng DNA from 16 adults.

8µl was loaded in each of the wells, corresponding to 1/100th of a total DNA preparation for each species,

The smearing at the bottom of the gel is RNA, not yet removed from the preparation.

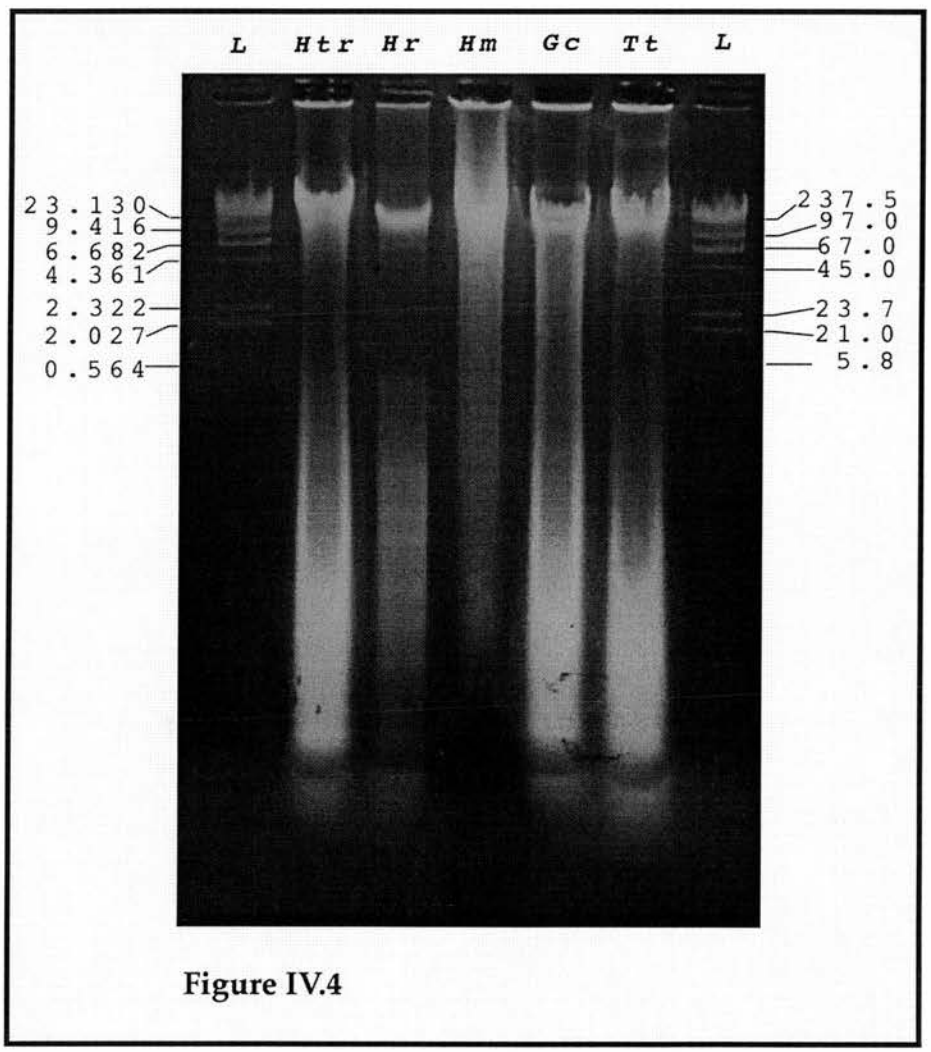
L
Htr
Hr
Hm
Gc
Tt
L
23.130
9.416
6.682
4.361
2.322
2.027
0.564
237.5
97.0
67.0
45.0
23.7
21.0
5.8

Figure IV.4

in *Drosophila melanogaster* yields two fragments of size 2.3kb and 4.5kb that hybridise with the THΔ1 probe. The different species used in this experiment were: *Drosophila melanogaster* (control), and 5 Hirudinea species from 2 different orders: *Hirudo medicinalis* (gnathobdellid order), *Theromyson tessulatum, Helobdella robusta , Helobdella triserialis* and *Glossiphonia complinata* (rhynchobdellid order, Glossiphoniidae family). I used the same amount of DNA (4μg) for each species on an agarose gel (see Figure IV.5). The gel was probed several times, using different hybridising conditions: I started with the most stringent conditions, lowering them until a signal appeared in the leech DNA lanes (for the different stringency conditions, see Methods).

In high stringency conditions, only the *Drosophila* lane showed a signal, as might be expected: the *h* gene is not 100% conserved between *Drosophila* and any of the leech species studied. The leech lanes showed only a high background which may have been due to yolk protein still attached to the DNA. At medium stringency, a very faint signal appeared in the *Theromyzon* lane around 4 kb (see Figure IV.6). The band did not appear any stronger at very low stringency, and even in the other species, no signal appeared.

However, this was considered sufficient indication that the gene might be present and I went on to use PCR to detect and clone *h*.

After the PCRs gave no evidence of the presence of *h* (see below), I did another Southern blot, this time using the more sensitive technique of chemoluminescence at very low stringency. This was to test whether the signal previously observed was true or just background in *Theromyson.* The second Southern blot was run in the same conditions as the first Southern blot, at lowest stringency (see Methods). This time unfortunately, the DNA extracted from *Theromyson tessulatum* was slightly degraded (see Figure IV.7A). However, the DNA from the other species, was of good quality, and

Figure IV. 5 The agarose gel used for Southern blotting. The DNA has been cut with EcoRI (compare to Figure IV.4). Each lane was loaded with approximately the same amount of DNA (4µg). The size marker is the 1kb ladder, in the first lane. Corresponding sizes are in kb, to the left of the first lane.*(Ht):Helobdella triserialis ; (Hr): Helobdella robusta; (G.c.): Glossiphonia complinata; (T.t):Theromyzon tessulatum ; (Hm): Hirudo medicinalis; (Dm): Drospophila melanogaster.*

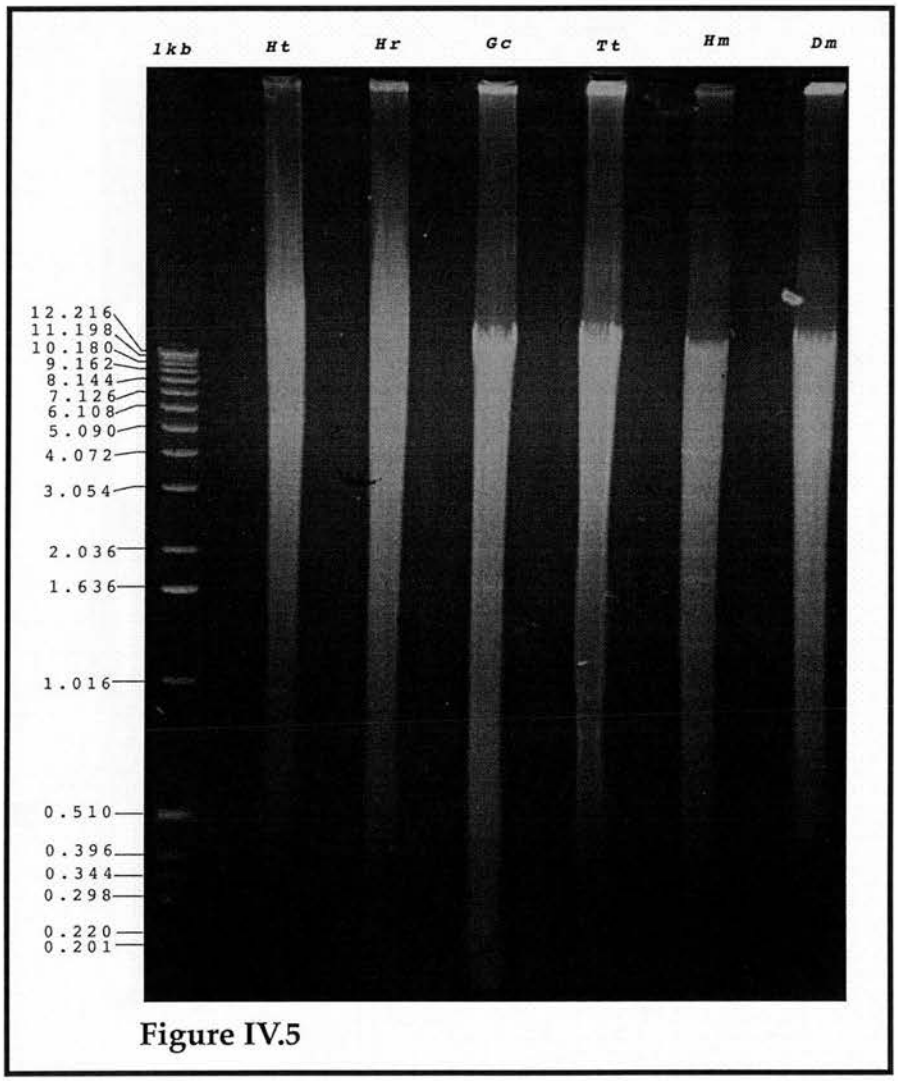
1kb
Ht
Hr
Gc
Tt
Hm
Dm
12.216
11.198
10.180
9.162
8.144
7.126
6.108
5.090
4.072
3.054
2.036
1.636
1.016
0.510
0.396
0.344
0.298
0.220
0.201

Figure IV.5

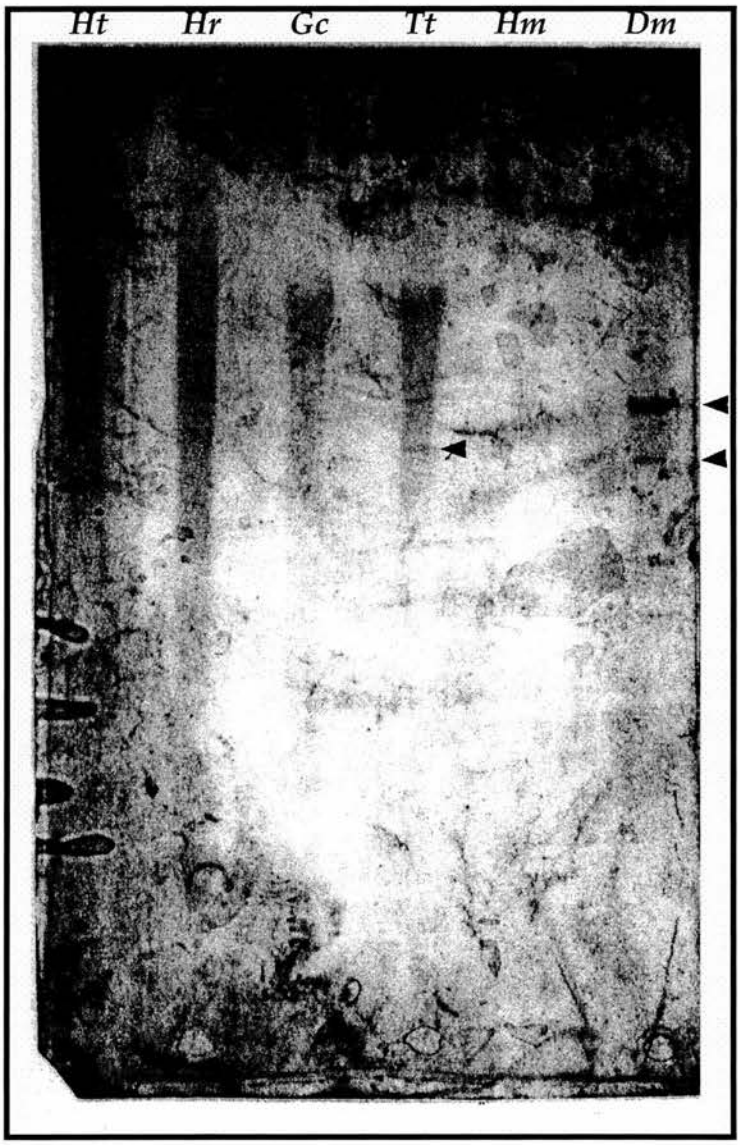

Figure IV. 6 The Southern blot, hybridised with the THΔ1 probe. see figure 3 for the original agarose gel. Two bands appear in the Drosophila lane, size 4.5 kb and 2.3 kb (arrows). One faint band appears in the *Theromyson tessulatum* lane, at about 4 kb (arrow). *(Ht):Helobdella triserialis ; (Hr): Helobdella robusta; (G.c.): Glossiphonia complinata; (T.t):Theromyzon tessulatum ; (Hm): Hirudo medicinalis; (Dm): Drospophila melanogaster.*

this case, only the control *Drosophila* gave a signal (see Figure IV.7B): no signal was detected in either species of leech at low stringency.

Overall, the results of the Southern blots failed to provide convincing evidence for a highly conserved h homologue.

PCR: on the Library cDNA, genomic DNA and on RNA extracts

Introduction

I used degenerate primers that had already been used for cloning a variety of basic-Helix-Loop-Helix molecules, including *h* in a variety of species (Domingos Henrique, unpublished results; see Figure IV.3). The *hairy* protein is a Helix-Loop-Helix protein that has homologies to the *enhancer-of-split [E(spl)]* family of genes (Delidakis & Artavanis-Tsakonas, 1992, see Figure IV.2). The primers were degenerate enough to allow for cross-reactivity. Most notably, both the *h* and *E(spl)* families have the WRPW motif at their C terminal end, recognised by the W primer (see Figure IV.1, 2, and 3).

The different primers were used in the different possible combinations: 1 and W (refered to as 1/W), 3 and W (3/W), 4 and W (4/W), 4 and D (4/D), 1 and D (1/D), 4 and A (4/A), and 1 and A (1/A) (see Fig. IV.3 for the sequence of the primers, and the positions of the primers relative to the protein sequence in *Drosophila*).

I amplified *Helobdella robusta* genomic DNA by using several degenerate oligonucleotide primers homologous to regions including the bHLH domain (primers 1, 3, 4, A, B and D) and a region downstream of it (the W primer). The *h* bHLH domain in *D.melanogaster* is interrupted by two introns, so I also amplified *h* sequences from either a cDNA library (stages 8 to 11) or from embryonic cDNA of various stages. The W primer recognises the invariant WRPW amino-acid pattern at the C terminal end of the protein. However, the distance between the Helix-Loop-Helix domain

Figure IV.7 (A) Agarose gel and **(B)** Southern blot, using the chemoluminescent technique. The lanes are aligned for comparison between the gel and the autoradiograph. arrows show the 4.5 and 2.3 kb fragments in Drosophila. The only intensity detected in the blot comes from background. (Dm, *Drosophila melanogaster;* Tt, *Theromyzon tessulatum*; Gc, *Glossiphonia complinata*; Ht, *Helobdella triserialis*). No size markers appear, but the sizes in *Drosophila* could be estimated on the basis of previous similar Southern blots.

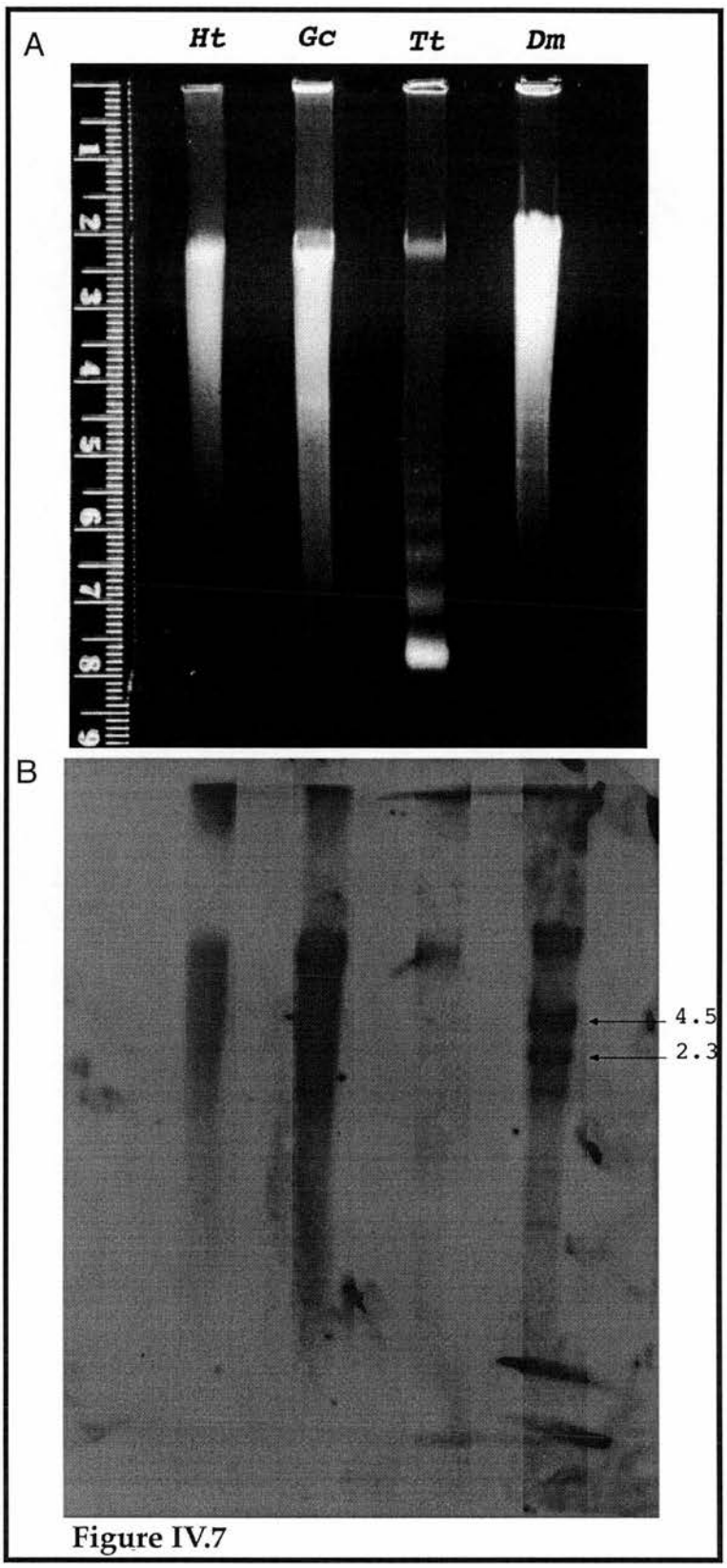

Figure IV.7

and the WRPW domain varies between the different families of b-HLH proteins (see Figures IV.1 and 2).

PCR on genomic DNA

I used the DNA available at the time: *H.robusta* DNA (0.75µg) and *H. medicinalis* DNA (1µg) and ran the PCR at low stringency conditions (see Methods) with the combination of primers 1/A (Figure IV.8a).

A faint band appeared around 220 bp in the *H. robusta* reaction. No negative control was run at the same time. However, no band appeared in the *H. medicinalis* or in another PCR run in parallel on unpurified *H. robusta* library DNA (2000 genome copies equivalent).

The experiment was repeated in H. robusta, still at low stringency, using 5 times more starting DNA and a positive control (*engrailed*, see Methods) for checking the quality of the DNA (Figure 8b). A band appeared around 220 bp but many other bands appeared too. Due to possible introns since this was genomic DNA, it was not possible to predict which band (if any) was likely to be a hairy fragment. Because of this and because of the background (due to the low stringency conditions), no band was cloned.

I used cDNA instead of genomic DNA as a template in the further PCRs.

PCR on cDNA from the library:

I first used the library unpurified by amplifying directly on the phages after a hot start for denaturing the DNA. The embryonic *H.robusta* library was titered at $2.5\ 10^{10}$ pfu/ml (plaque forming units/ml), and I used 1µl i.e. $2.5\ 10^{7}$ pfu per 50 µl reaction. The amplification was done at low stringency (see Methods). The A/1 combination of primers gave many bands, among which there was an intense one at 120bp (see Figure IV.9), as predicted with this set of primers (see Figure 3A). However, because of the high number of bands amplified, I decided to do a more stringent amplification before isolating the amplified fragments for cloning.

Figure IV. 8 (A) PCR using the primers 1 and A on *H. robusta* (*Hr)* genomic DNA, *H. medicinalis* (*Hm*)genomic DNA, and *H. robusta* unpurified cDNA library (L), starting with 0.75µg of DNA, 1µg of DNA and 2000 copies respectively. A single, faint, band appears at 220bp in *Hr.*(arrow). No negative control was run (water instead of DNA) but there is no band in the Hm and L lane, suggesting that there is no contamination (the high intensity at the bottom of the gel is due to the non incorporated primers). 1/5th of the total PCR reaction was loaded. The size marker was the 1kb ladder. The sizes are in kb. **(B)** Similar PCR using 5 times as much DNA as in (a), repeated only on *H. robusta (2)*, and with a positive control *(1)* using the *engrailed* primers 879 and 880 on *H. robusta* DNA. This time many bands appeared, among which there is a band at around 220 bp (arrow), but there was too much background for cloning it. A band appears at 120bp (gray arrowhead) in the control as expected (see Fig. IV.10B); more bands are present also, due to the lower stringency conditions. 1/5th of the total PCR reaction was loaded. Size markers were Φx (*F)*; the sizes are in kb.

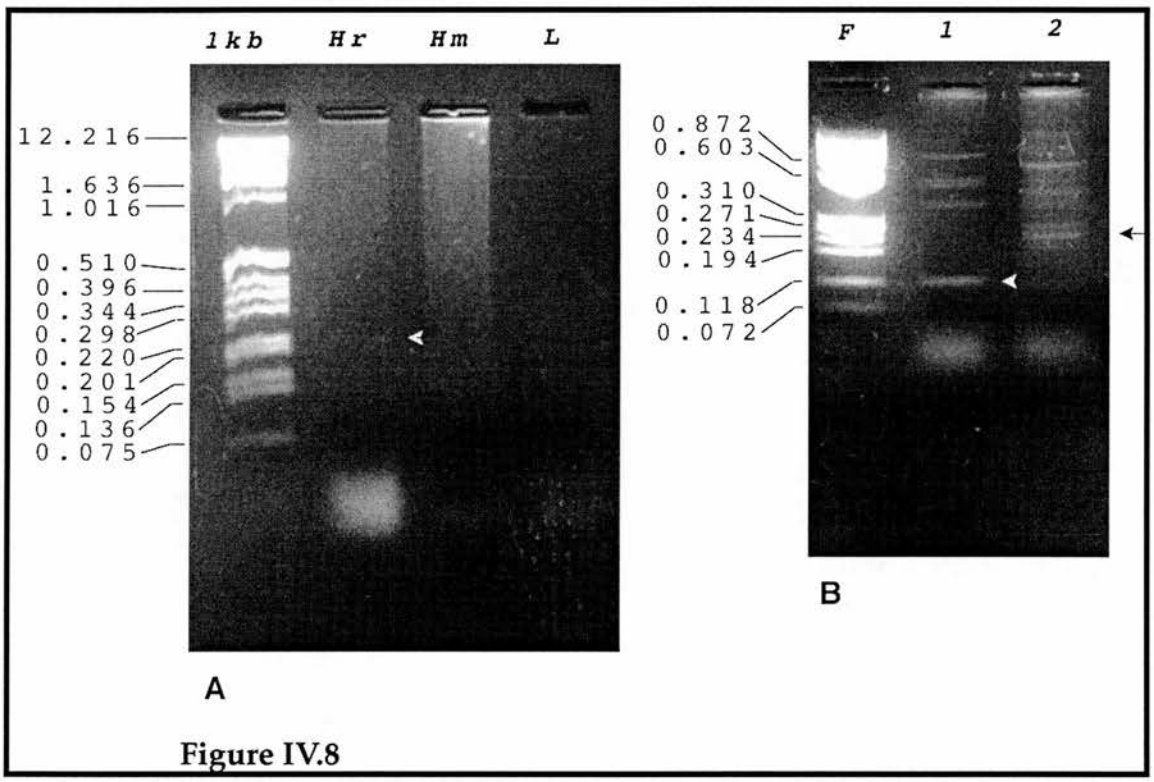
1kb
Hr
Hm
L
12.216
1.636
1.016
0.510
0.396
0.344
0.298
0.220
0.201
0.154
0.136
0.075
A
F
1
2
0.872
0.603
0.310
0.271
0.234
0.194
0.118
0.072
B

Figure IV.8

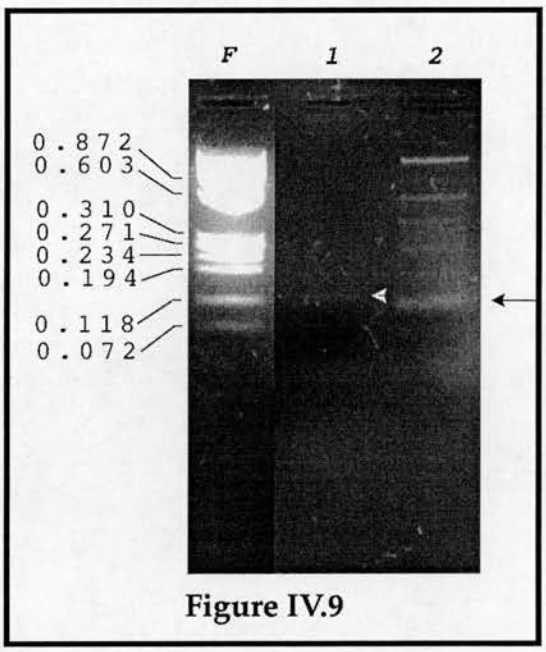

Figure IV.9

Figure IV.9 Result of PCR using the combination 1/A of primers on the H. robusta cDNA library, unpurified, with $2.5x10^7$ pfu as starting material: *F*: Φx size markers, (*1*): control PCR using primers 879 and 880 on the library, (*2*): PCR using the primers A and 1 on the library. A band appeared around 120bp (arrow), but many others appeared too, due to the low stringency of the reaction. The control reaction shows a very faint band around 220bp. Sizes are in kb.

I purified the DNA from the *H. robusta* library in order to obtain better amplification. In a control experiment, I used a primer designed against the *twist* gene in combination with a T3 primer (to the library plasmid), and the primers 879 and 880 against *en.* (see Methods). When these showed satisfactory bands at the expected sizes, at 300bp (*twist*, see Figure IV.10A) and 120 bp (*en*, see figure IV.10B) respectively, I went on to amplify the library using the *h* primers. I first restrained my search by using combinations 3/W, 4/W, 4/A and 4/D on the library and obtained a large number of bands (see Figure IV.11). As mentioned earlier, the size of the fragments amplified by using the W primer could not be predicted because this is expected to change from one species to the other, certainly from one bHLH gene to another (Wainwright & Ish-Horowicz, 1992). We know, however, that with the combinations 3/W and 4/W, approximately the same size of band should be amplified, with the 4/W generated fragment being larger by 111 bp (in cDNA amplifications). I used this as a guideline in analysing the resulting gels run after the PCR, and for deciding which bands to clone. With this in mind, the following bands were cloned (see gel and interpretation in Figure IV.11): 3/W 400 bp, 4/W 1kb, 4/W 500 bp, 4/W 400 bp, 4/A 140 bp, 4/D 130 bp.

These were cloned and sequenced with the following result: the 4A and 4D clones (a minimum of 3 of each) yielded sequences 100% homologous to the *Drosophila melanogaster* sequence. This probably resulted from contamination. This was tested by running running PCRs with water instead of DNA, and using the different combinations of primers. Only the reactions in which the primer 4 was used was there a band consistent with Drosophila cDNA contamination. A new batch of primer 4 was tested by doing two rounds of PCR using only water as a template. This time, no band was amplified.

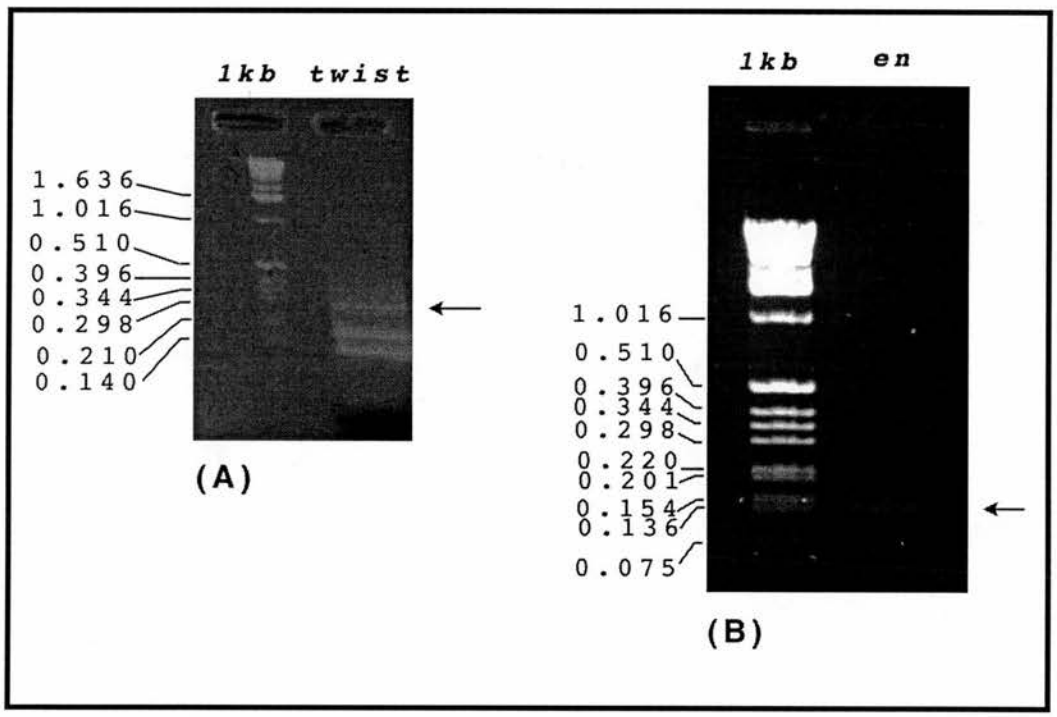

Figure IV. 10 (A) PCR control on the cDNA library extracted DNA, using the primers Twist and T7. A band appears at about 300 bp as expected (Julio Soto, personal communication), but the smaller band are not specific. (*Twist*: PCR, *1kb*: 1 kilobase ladder marker). The sizes are expressed in kb. **(B)** The control PCR used the primers 879 and 880, targeted at the *en* gene. Arrow points at the 120 bp band resulting from the PCR.(*en*: PCR, 1*kb*: 1kb ladder). The sizes are expressed in kb.

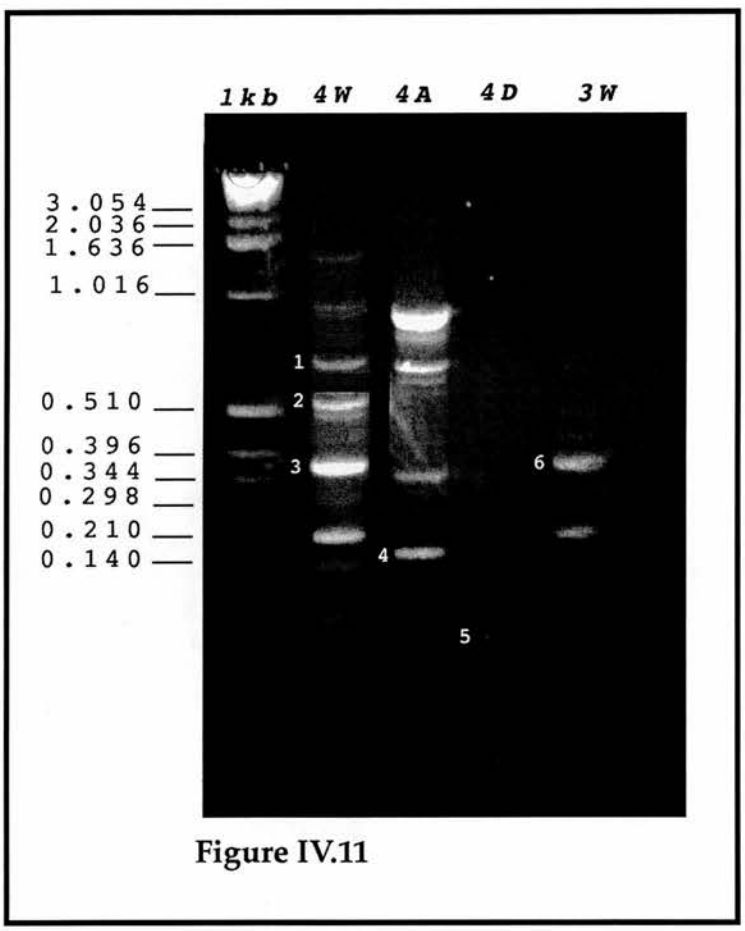

Figure IV.11

Figure IV. 11 PCR on the *H.robusta* cDNA library, using the primer combinations 4 and W (4W), 4 and A (4A), 4 and D(4D), and 3 and W (3W). The size marker was the 1kb ladder (1kb). The fragments that were later isolated and cloned are numbered to their left as follows:
1: 800bp fragment from the 4W PCR
2: 500bp fragment from the 4W PCR
3. 350bp fragment from the 4W PCR
4: 140 bp fragment from the 4A PCR
5: 130 bp fragment from the 4D PCR
6: 350 bp fragment from the 3W PCR

All the clones which contained inserts that had been amplified using the W primer had the characteristic of containing the W primer sequence at both ends of the clone. None contained the HLH domain as would have been expected from my primers (1, 3, 4, B and D all recognise a region within the HLH domain). A consensus sequence was, however, found among a majority of these clones, sequenced only up to about 150bp on each strand (see Figure IV.12A). When the sequence was entered and compared with the GCG database, it showed no homology to any known sequence. This part of the molecule is not conserved, so it is not possible to decide whether the fragment is part of a *hairy* homologue or not.

To decide whether it was possible that the fragment was part of the *h* gene, I compared the sequences obtained from the different clones that res embled each other. It was possible to obtain a consensus amino-acid translation without stop codon, suggesting the RNA could code for a protein (see Figure IV.12b). However, while clones of different lengths gave the same sequence in the first 100bp, sequencing from both ends never produced the sequence of the complementary primer (i.e. primer 1, 3 or 4). Therefore the clones sequenced have a conserved WRPW domain, but no conserved bHLH domain: it is unlikely that they are *h* homologues.

I subsequently ran a control PCR in which only one primer, the W primer, was present. Bands appeared at different lengths, confirming that the W primer could be used by the template DNA as both a forward and a backward primer (see Figure IV.13) .

Nested PCRs were also attempted in which the first round of amplification contained one primer to the library plasmid (i.e. T3 or T7 primer) and one plasmid specific for the *h* gene, i.e. 4, 3, D and W, in the 8 different possible combinations (because the orientation of insertion is not known), and a second round using two specific primers. After the first round of amplification already, only the amplifications using the W primer

(A)

```
     1                                                            50
H14  CCTGGGGCGC CAGTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT
H15  CCAGGGGCGC CAGTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT
1_3w CCAGGGGCTC CAGTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT
H23  CCAGGGGCGC CAGTTTTCTG TTGATGCTGC AAGAAGTTCC TCGTAACTCT
H25  CCAGGGGCGC CAXTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT
H27  CCAGGGGCGC GAGTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT
3_3w CCAGGGGCGC CAGTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT
3w1  CCAGGGGCGC CAGTTTTCTG TTGATGCTGC AAGAAGTTGG TCGTAACTCT

     51                                                           100
H14  GCTGCGCGCX CTTTTCAAGC TGGCGAACGA CGCGCTGCCC CACTCGTCTA
H15  GCTGCGCGCA CGTTTCAAGC TGGCGAACGA CGCGCTGCCC CACTCGTCTA
1_3w GCTGCGCGCA CATTTCAAGC TGGCGAACGA CGCGCTGCCC CACTCGTCTA
H23  GCTGCXCXCA CATTTCAAGC TGGCGAACGA CXCCTGCCCC ACTCGTCTAA
H25  GCTGCXXCAC ATTTCAAGCT GGC..... ..........  ..........
H27  GCTGCXXCAC ATTTCAAGCT GGCXAAGACX XCTGCCCCAC TCGTCTAAAA
3_3w GCTGCGCGCA CATTTCAAGC TGGCGAACGA CGCGCTGCCC CACTCGTCTA
3w1  GCTGCGCGCA CATTTCAAGC TGGCGAACGA CGCGCTGCCC CACTCGTCTA

     101                                                          150
H14  AAGCAGCTTG CCTGTCATCC CGCCAG....  ..........  ..........
H15  AAGCAGCTTT GCCTGTATCC CGCCAGCTGA CCCACACCAG  ..........
1_3w AAGCAGCTTG CCTGTCATCC CGCCAGTCGA TCCACACCAG TTTTTGCAAC
H23  AGCAGCTTGC CTAGCATCCC GCCAGTCGAT CCACACCAGT TTTTGACAAC
H25  ..........  ..........  ..........  ..........  ..........
H27  CAGCTT....  ..........  ..........  ..........  ..........
3_3w AAGCAGCTTG CCATGTCATC CCGCCAGTCG ATCCACACCG ATTTTTGCA.
3w1  AAGCAGCTTG CTGTATCCGC CAGTCGATCC ACACAGTTTT GCACGTATGT

     151       162
H14  ..........  ..
H15  ..........  ..
1_3w GTATTCGTCT  CT
H23  GTCATTGTCT  C.
H25  ..........  ..
H27  ..........  ..
3_3w ..........  ..
3w1  GTG.......  ..
```

Figure IV.12 Sequences of the clones obtained by PCR reaction using the primer combinaison 4W or 3W on the cDNA library. **(A)** DNA sequence; **(B)** deduced amino sequence. The consensus sequence is in bold.

(B)

```
(14)   R P R W N E T S A A L L Q D Y S Q Q A ? -
(15)   W P R W N E T S A A L L Q D Y S Q Q A C -
(23)   W P R W N E T S A A L L E E Y S Q Q ? ? -
(25)   W P R W ? E T S A A L L Q D Y S Q Q ? V -
(27)   W P R S N E T S A A L L Q D Y S Q Q ? V-
1_3w   W P S W N E T S A A L L Q D Y S Q Q A C -
3_3w   W P R W N E T S A A L L Q D Y S Q Q A C -
3w1    W P R W N E T S A A L L Q D Y S Q Q A C -


(14)   K E L Q R V V R Q G V R R F C S A Q * G -
(15)   T E L Q R V V R Q G V R R F C S Q R Y G -
(23)   M E L Q R V V G A G S T * L L K G L M G -
(25)   N * A P -
(27)   N * A P ? S ? Q G V R R F C S -
1_3W   M E L Q R V V R Q G V R R F C S A Q * G -
3_3w   M E L Q R V V R Q G V R R F C S A M D D -
3w1    M E L Q R V V R Q G V R R F C S A T D A -


(14)   A L -
(15)   A L Q G V G -
(23)   G T S G C W N K V V D N D
(25)
(27)
1_3w   A L R D V G T K A V Y E D R -
3_3w   R W D I W V S K Q -
3w1    L R D V C N Q V Y T H -
```

Figure IV.12 Sequences of the clones obtained by PCR reaction using the primer combinaison 4W or 3W on the cDNA library. **(A)** DNA sequence; **(B)** deduced amino sequence. The consensus sequence is in bold.

Figure IV.13 Control for the W primer. In a first PCR reaction, cDNA was amplified using the combination of primers 3W or 4W. Bands were purified and reamplified using either the same primers (a lanes) or, as a control, only the W primer (b lanes). Lane 1: template was a 350bp band purified from a 3W PCR; Lane 2: template was a 250bp band purified from a 3W PCR; lane 3: template was a 200bp band purified from a 3W PCR; lane 4: template was a 150bp band purified from a 3W PCR; lane 5: template was a 700bp band purified from a 4W PCR; lane 6: template was a 200bp band purified from a 4W PCR.
In reaction 1 and 2, there is as much amplification whether the forward primer (primer 3) is present or not. In reactions 3, 5 and 6, the amplification is actualy stronger if only the downstream primer (W) is present! Only in the reaction 4 is there only amplification when both primers (3 and W) are present.

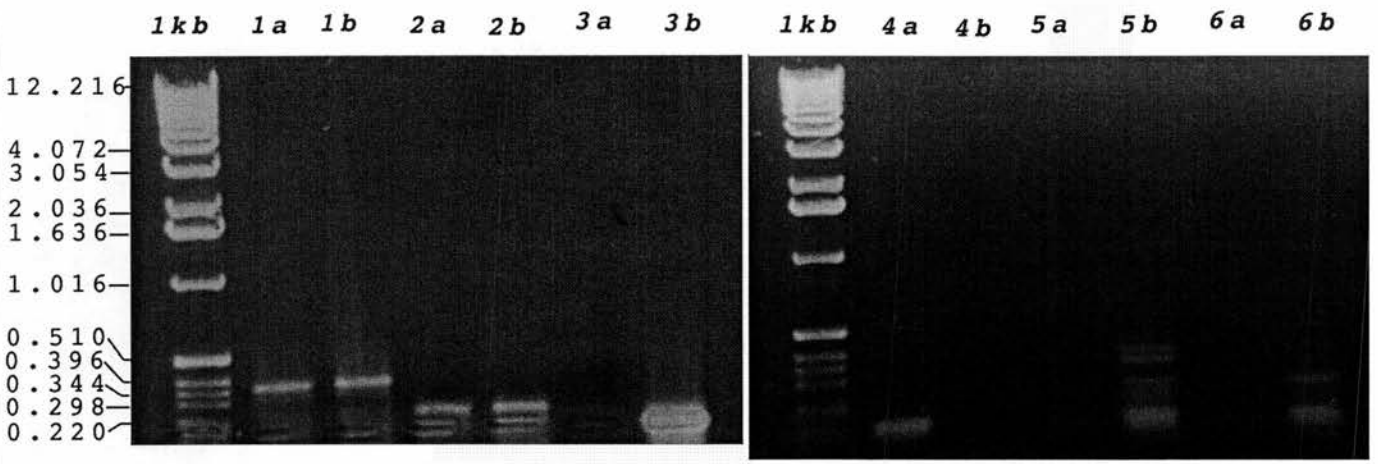
1kb
1a
1b
2a
2b
3a
3b
1kb
4a
4b
5a
5b
6a
6b
12.216
4.072
3.054
2.036
1.636
1.016
0.510
0.396
0.344
0.298
0.220

Figure IV.13

produced bands, confirming that only the W primer recognises a sequence in the library DNA (see Figure IV.14). Subsequent nested PCR suggested that it was a "W primer artifact", as previously. To check this, I amplified using the 4/D combination of primers in the second round of amplification on both W/T3 and W/T7 first amplification (see Figure IV.15). This did not give the expected band at 130bp; therefore, the fragments amplified by W/T3 and W/T7 did not contain a *h* fragment (i.e. conserved in the bHLH domain).

No obvious *h* homologue was cloned by PCR on the cDNA library (stage 9 to 11). It is possible that the library did not contain the required gene (see Discussion for more possible explanations).

However, a fragment was cloned, which contained a conserved WRPW motif, and with no resemblance to any known gene.

PCR on cDNA from extracted RNA from different embryonic stages.

Since I suspected that the *h* gene might be absent from library, I used mRNA extracted from different embryonic stages as a template for RT-PCR (reverse transcription polymerase chain reaction).*Theromyson* was used preferentially, because the embryos are larger and it is easy to get many synchronous embryos: one adult gives a few (1 to 4) cocoons of at least 30 embryos. One such batch could be used for an RNA extraction. Different types of first strands were synthesised, by using different types of primers (see Figure IV.16). The first strand chosen for subsequent PCR was polymerised using a mixture of hexamers for random priming.

A band was obtained at 600bp with the combination 4/D and 4/A on the juvenile stage cDNA, and was cloned and partially sequenced. This sequence (see figure IV.17) was unrecognised on the GeneBank (using MAP and FASTA on the GCG program), and did not match either primer 100%.

Figure IV. 14 Single-specificity PCR. **(1)** T3/3, **(2)** T3/4, **(3)** T3/D, **(4)** T3/W, **(5)** T7/3, **(6)** T7/4, **(7)** T7/D, **(8)** T7/W. Only the PCRs using the W primer resulted in amplification (arrowheads). The bright intensity at the bottom of the gel results from the unincorporated primers.

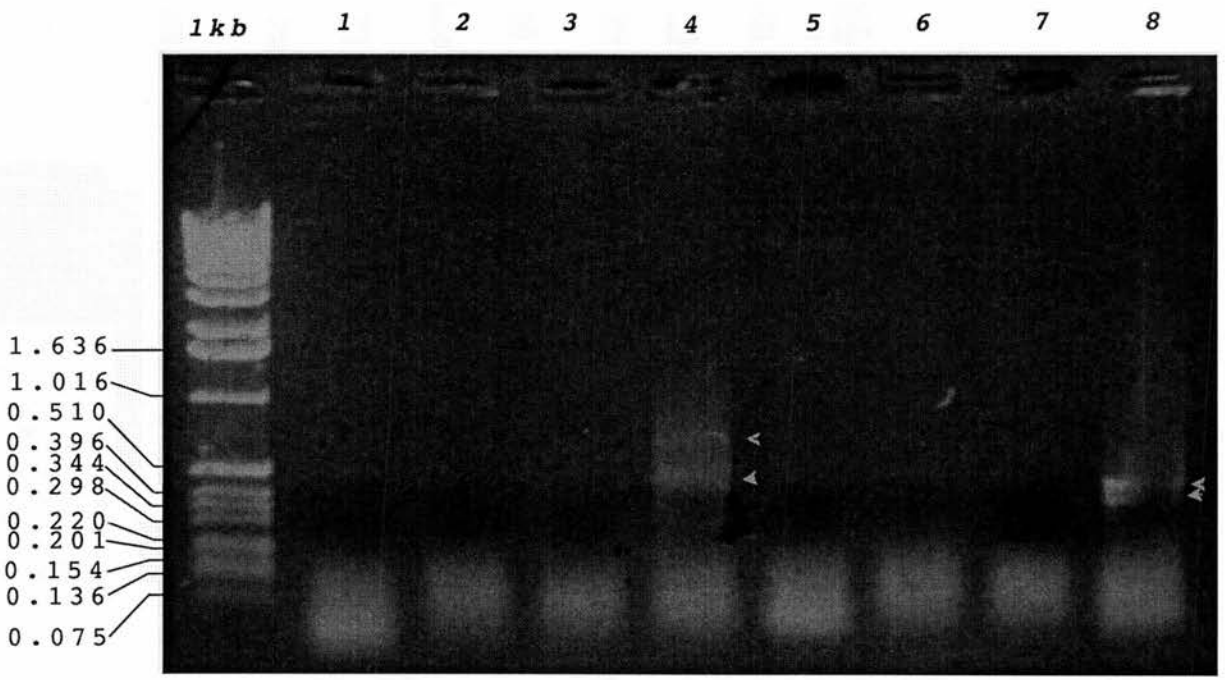
1kb
1
2
3
4
5
6
7
8
1.636
1.016
0.510
0.396
0.344
0.298
0.220
0.201
0.154
0.136
0.075

Figure IV.14

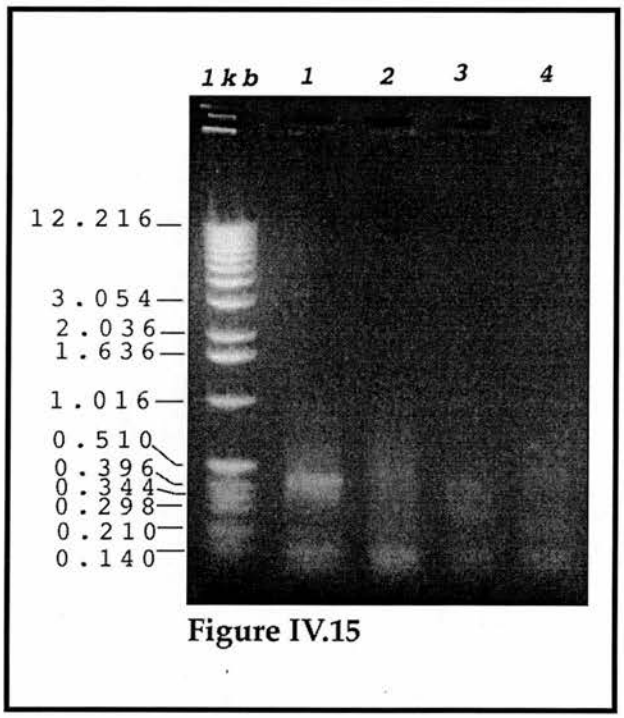

Figure IV.15

Figure IV. 15 Nested PCR on cDNA library from *H. robusta:* the first round of PCR ("enriching step") was done with the combination of primers T7 and W **(1)**, or T3 and W **(2).** Some amplification was visible, although a smear suggests a lack of specificity. In the second round of PCR ("specific step"), using the combination 4/D on either the PCR 1 **(3),** or the PCR 2 **(4)**, no band appeared.

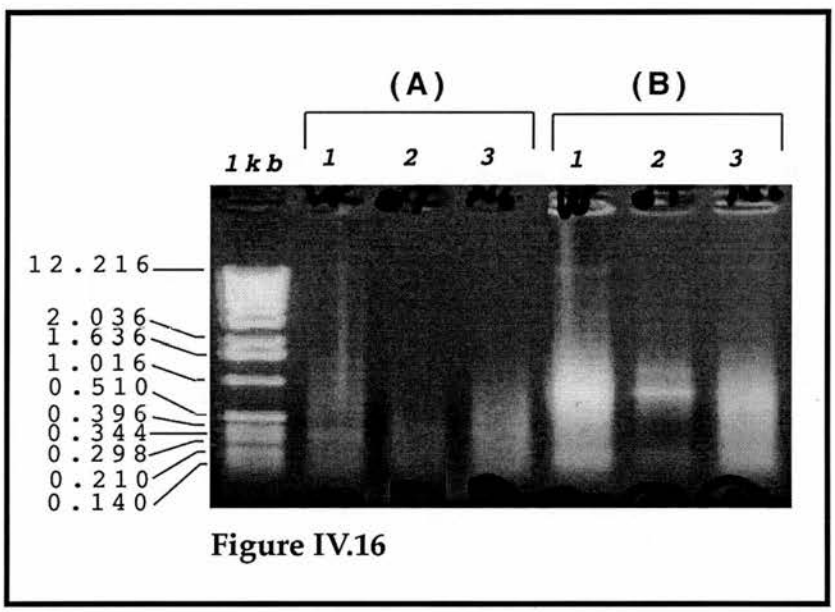

Figure IV.16

Figure IV. 16 Result of reverse trancription on total RNA extracted from stage 8 embryos **(A)** or juveniles **(B)**, using different primers: W primer (**1**), poly-dT primer (**2**), or random priming, using a hexanucleotide mixture (**3**). All reactions show a smear indicating fragments of various sizes with little degradation.

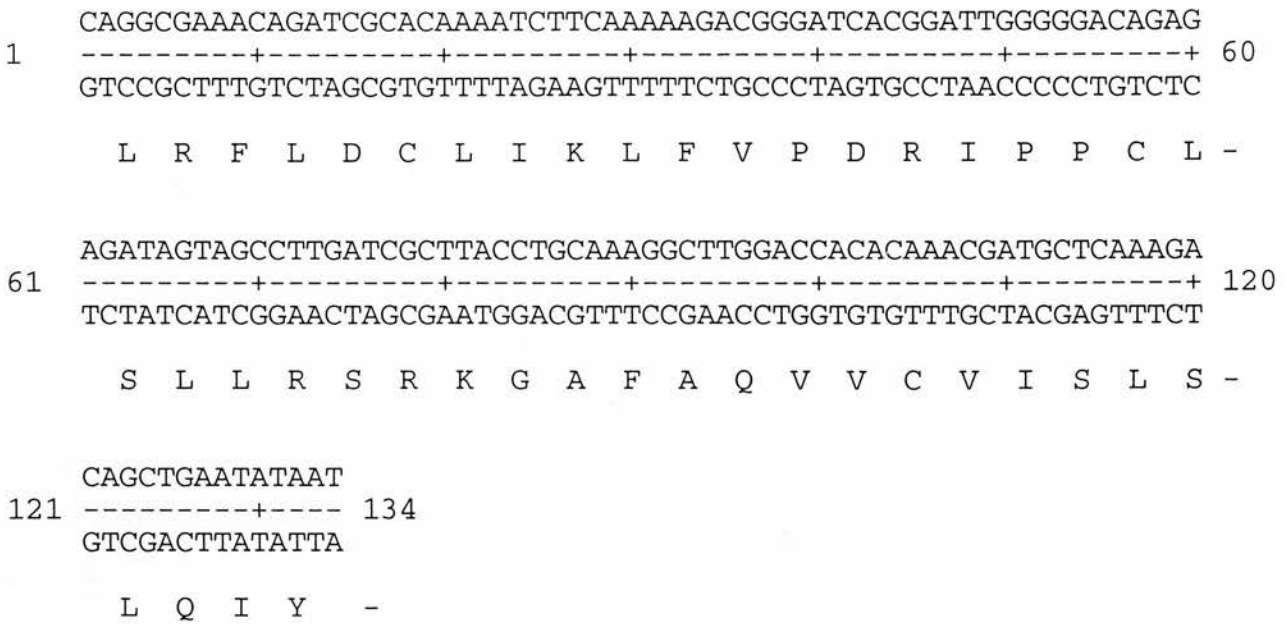

Figure IV.17 sequence from the 600 bp PCR fragment, obtained using the combination of primers 4 and D on cDNA of juvenile stage *T. tessulatum* . The line below the DNA sequence (sense and antisense) is the virtual transltion into aminoacids.

However, it contained an open reading frame in each direction, and it is possible that it is part of a novel gene.

The 136bp band was recovered and cloned, yielded a sequence 100% homologous to the *Drosophila*. This was contamination as for the PCR on the cDNA library.

Discussion: library screening and other possible methods - a perspective

The two different approaches, Southern blotting and PCR, have led to inconclusive evidence for the presence of the *h* gene in a form closely related to the *Drosophila h* in the species of leech studied here.

One Southern blot suggested there may be a homologue, the other that there may not be.

One Southern blot suggested there may be a *h* homologue in the leech (in 1 of the 5 species of leech studied), but the most sensitive Southern blot suggested there was not (in 2 species of leeches studied). Technical problems, such as high background were encountered. This is a common problem in the leech (Wedeen, et al., 1990b), which meant that the total amount of DNA had to be restricted, and that a faint signal might be undetected among the background. The genome of *Helobdella* (about 1.5 x 10^6 kb, Wedeen et al., 1990) is approximately 2.5 times larger than the genome of *Drosophila* (0.6 x 10^6 kb, Rash et al., 1971). Therefore, considering that approximately the same amount of*Drosophila* and *Helobdella* DNA was loaded on the gel, it is unlikely that the amount of DNA in the *Helobdella* lanes was too small to be detected. This, however, only suggests that the gene might be divergent in different aspects: introns might be present that impair the binding of the THΔ1 probe (cDNA probe); the secondary structure of the DNA may be a hindrance to hybridising a divergent sequence.

PCR led to cloning of contaminants and unrelated sequences

The only evidence for a possible *h* related gene obtained in the PCR was obtained when using genomic DNA as a template. A band was consistently obtained at 220bp at low stringency, while the negative control showed it was unlikely that there was any contamination. But the nature of the band was not determined, and although it is possible that this band reflects the presence of a *h* homologue (with an intron of 100bp), this cannot be determined on the sole basis of a band. In effect, bands were obtained in the different PCR that were either not *h*-like nor contaminants.

In the library, technical problems (contamination) led to the cloning of a *Drosophila* fragment. However, nested PCR with un-contaminated fragments produced no band, suggesting that the gene is either not in the library or is divergent at the primer sites. Different primers led to the cloning of fragments unrelated to *h*.

RT-PCR led to the cloning of another un-related fragment and to contamination, leaving no evidence for or against the presence of a *h*-related gene.

Further work

More work could involve choosing different primers (e.g the primers used in Sommer and Tautz, 1993 for PCR in *Tribolium*, or screening a cDNA library with the THΔ1 probe. This screening would have the advantage that it would rest on different homology properties than the PCR for detection, and eliminate the difficulty possibly posed by introns in the Southern blot.

What the absence of a h *homologue in the leech could mean*

However incomplete, these results tend to suggest the absence of an *h* gene in the leech. It is therefore possible to argue that the *h* sequence has diverged enough from the common ancestor of leech, *Drosophila*, and vertebrates to make it unrecognizable by Southern blot and PCR.

I have argued in the Introduction that *Drosophila* and leech segment in a different manner: it is only at the stage of the *en* expression that the two organisms start showing similarities of segmentation, by expressing the segment polarity gene in a striped pattern, one stripe per segment. In the leech, the founder cells of the segments are born with an identity, as argued by the expression of a homeotic gene, *lox2*: this gene is expressed in the different cells long before segmentation is apparent and before the different cells founding the same segment come into register (Nardelli-Haefliger et al., 1994). The blast cells might contain in their genetic program, endowed at birth or soon after, the information for the axis of their cell division, and positional information that will allow them and their descendents to form coherent segments.

Ablation of a blast cell, founder of part of different segments, results in the absence of the structures normally generated by this founder cell: there is regulation of the defect only exceptionally (Martindale and Shankland, 1990). All this suggests that segmentation might not require the positioning of boundaries by cell communication.

What about the final boundary, the 32nd/33rd segment boundary? I have argued that some external signal is required for the setting up of that boundary. However, there are different candidate molecules, the most obvious being other pair-rule genes such as *eve* or *ftz*. Other possible examples will be discussed in the final chapter.

If *h* was never used in the "limiting boundaries" process in the leech, it is probable that it has diverged beyond recognition, had it been present in the common ancestor of the arthropods and the annelids. We can therefore make no assumption on evolution. In effect, the fact that a *h*-related gene is found in vertebrates (Ishibashi et al., 1993; Sasai et al., 1992) and not in annelids could prompt the idea that arthropods are more closely related to vertebrates than to annelids (see Figure IV.18.). However, if it is through a

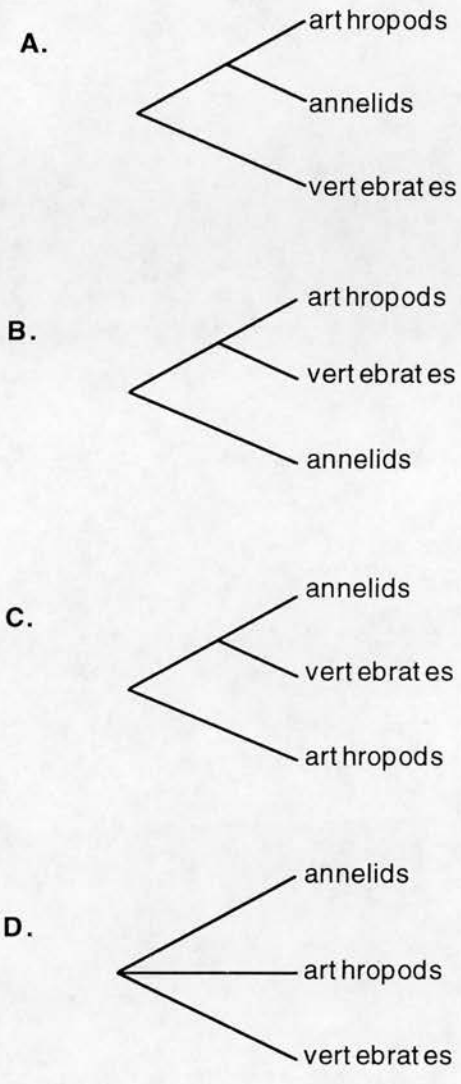

Figure IV. 18 The different possible phylogenetic relationships between arthropods, vertebrates, and annelids. The most favored tree in classical zoology is the tree A. (Hyman, 1951); molecular studies favour different phylogenies: A. (Lake, 1990) or C. (Field et al, 1988).
If the separation of the different phyla was very close in time, it is possible that with our limited perspective (whether using molecular evolution or morphological and anatomical characteristics), the distinction could not be established and we would see the tree as D. In fact, Field's tree (C.) tends towards that view. This is assuming that arthropods are not polyphyletic, as was suggested (Anderson, 1973).

modification of developmental mechanisms (of the first stages leading to a segmented body) in the annelids that the *h* gene has become disused, then we can argue that this would have prompted a faster divergence of the gene than in the species where the *h* protein was still being used as a transcription factor, in arthropods and in vertebrates for example. The fact that *eve* does not serve a pair-rule gene role in short germ-band insects (Patel et al., 1992) gives weight to the hypothesis that the pair-rule pre-pattern would have become disused in the descendents of a common pair-rule pre-patterned animal, of which both annelids and short-germ band insects would be.

Two novel genes in the leech?

As a by-product of this research, I have cloned novel PCR fragments amplified from a *H.robusta* embryonic cDNA library. Although these two fragments could not be related to any known sequences, there is the possibility that these fragment are part of novel genes. In order to ascertain whether this is the case, rather than these fragments being PCR artifacts, a genomic or cDNA library could be screened with these cloned PCR fragments to try and obtain a sequence of coding region.

Conclusions

At this moment, no evidence for a *h* gene in leeches has been found, but further experiments might uncover a divergent gene. The PCR has led to the uncovering of sequences that cannot be related to any other known sequence in the leech or in other species. Future work could tell us whether these code for part of a novel protein.

Chapter V: Pattern of expression of a putative *patched* gene in the leech embryo

Introduction

The segment polarity genes are expressed in segmental periodicity in the blastoderm of Drosophila from the beginning of gastrulation. They act to maintain and refine the borders established by the pair-rule genes (Nüsslein-Volhard & Wieschaus, 1980). Some of the segment polarity genes encode transcription factors, but the remainder are involved in intracellular communication and intercellular signal transduction. Some have been found in other systems where signalling is required for patterning (Ingham, 1991). There is also evidence that these genes might be involved in signalling in species as distant from the insects as the verebrates (Krauss et al., 1993)

If, as I have argued (Chapter IV), the counting problem consists of setting a border between the posterior end of the last segment and the anterior of the unsegmented tissue, then segment polarity signalling molecules are likely candidates for setting up the boundary. Here, I look for the presence and expression pattern of one of the molecules involved in the *hedgehog (hh)* signalling pathway, *patched (ptc).*

Background to the hedgehog (hh) and wingless (wg) signalling pathways in development in the Drosophila

The segment polarity role.

Segmentation in Drosophila occurs by progressive subdivision and relies on the correct spatial and temporal expression of segmentation genes: maternal, gap, pair-rule, segment polarity (see Chapter I). As each set of genes is expressed, segments get more spatially defined (Ingham, 1988). The patterning within the segments is initiated by the expression of the segment

polarity genes. These genes comprise a heterologous group of genes including *wingless (wg), engrailed (en), patched (ptc), hedgehog (hh), naked (nkd), fused (fu)* (Ingham, 1993). The segment polarity genes are under the control of pair-rule genes, registering the positional cues generated by them. They are defined by their mutant phenotype in which there is disruption of the polarity of the segments (Nüsslein-Volhard & Wieschaus, 1980): in each segment, a defined fraction of the pattern is deleted and the remainder is a mirror-image duplication. In *wingless* and *hedgehog*,, the anterior margin of the duplicated region lies posterior to the boundary, such that these larvae apparently lack all segment boundaries. In *patched*, the duplicated structure involves structures of two adjacent segments. *Patched* larvae, despite the normal number of denticle bands, have twice the number of segment boundaries (Nüsslein-Volhard & Wieschaus, 1980).

These genes have been analysed molecularly and in terms of function, and it now seems that they are all part of two complementary pathways, with the function of setting up and maintaining segment boundaries. The Hedgehog protein is secreted by cells that express the *engrailed* (*en*) gene, at the anterior boundary of the parasegments. Hedgehog protein in turn induces the expression of the secreted signalling molecule Wingless in the posterior half of the parasegment. *wingless* espression is then required for the stabilisation of *engrailed* expression (see Figure V.1). The expression of the segment polarity genes is maintained after gastrulation when maternal, gap and pair-rule genes have decayed, and the cellular signalling system mediated by these genes continue to refine patterns within each segment (Patel 1994).

Comparative molecular studies have found that maternal, gap and pair-rule genes are not used for setting up segmentation in other phyla. In fact, only insects that develop in a very similar way to *Drosophila* (long germ band) express some of these genes in a pattern compatible with a

polarity genes. These genes comprise a heterologous group of genes including *wingless (wg), engrailed (en), patched (ptc), hedgehog (hh), naked (nkd), fused (fu)* (Ingham, 1993). The segment polarity genes are under the control of pair-rule genes, registering the positional cues generated by them. They are defined by their mutant phenotype in which there is disruption of the polarity of the segments (Nüsslein-Volhard & Wieschaus, 1980): in each segment, a defined fraction of the pattern is deleted and the remainder is a mirror-image duplication. In wingless and hedgehog, the anterior margin of the duplicated region lies posterior to the boundary, such that these larvae apparently lack all segment boundaries. In patched, the duplicated structure involves structures of two adjacent segments. Patched larvae, despite the normal number of denticle bands, have twice the number of segment boundaries (Nüsslein-Volhard & Wieschaus, 1980).

These genes have been analysed molecularly and in terms of function, and it now seems that they are all part of two complementary pathways, with the function of setting up and maintaining segment boundaries. The Hedgehog protein is secreted by cells that express the *engrailed* (*en*) gene, at the anterior boundary of the parasegments. Hedgehog protein in turn induces the expression of the secreted signalling molecule Wingless in the posterior half of the parasegment. *wingless* espression is then required for the stabilisation of *engrailed* expression (see Figure V.1). The expression of the segment polarity genes is maintained after gastrulation when maternal, gap and pair-rule genes have decayed, and the cellular signalling system mediated by these genes continue to refine patterns within each segment (Patel 1994).

Comparative molecular studies have found that maternal, gap and segment-polarity genes are not used for setting up segmentation in other phyla. In fact, only insects that develop in a very similar way to Drosophila (long germ band) express some of these genes in a pattern compatible with

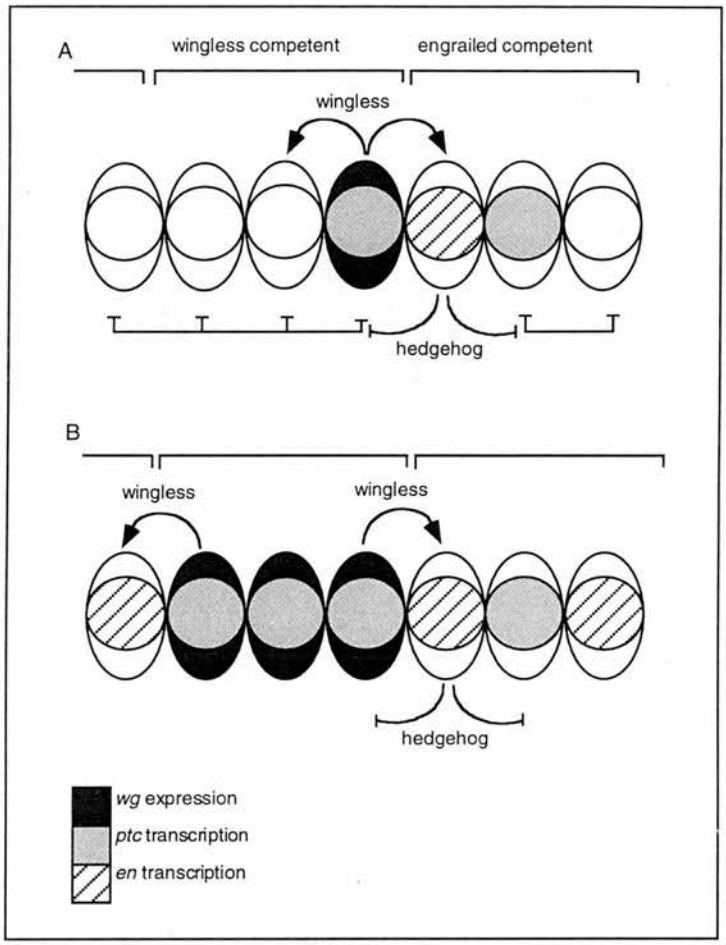

Figure V.1: a model for cellular interaction after gastrulation in *Drosophila*

A, *ptc* protein is initially expressed in all cells except for those expressing *en;* this expression serves to repress transcription both of *wg* and *ptc* itself, unless the activity of the protein is antagonised by *hh.* This results in the maintenance of *ptc* transcription in cells on either side of the *en* cell, but of only *wg* in cells anterior to the *en* cell, because only these are predisposed to express *wg.* Similarly, the *wg* signal maintains en expression, but only in cells posterior to the *wg* cell as these alone are predisposed to express *en.* B, In the absence of *ptc* activity, all cells, except those expressing *en*, now transcribe *ptc*, whereas all cells with the appropriate competence express *wg.* This results in the juxtaposition of cells predisposed to express *en* with *wg*-expressing cells, resulting in the ectopic induction of *en.*

segmentation role (Patel, 1994). On the other hand, studies of the segment polarity genes in a variety of organisms have revealed that they are present in a pattern and at a time compatible with segment patterning role. The best studied of these genes, *engrailed*, is conserved among other insects, crustacea, and annelids. In these animals that develop sequentially along an antero-posterior gradient, the expression of *engrailed* appears antero-posteriorly, and at a comparatively later stage than in *Drosophila*. They are, nevertheless, expressed in segmental periodicity, suggesting that they might play a role in establishing segmental borders.

wg and *hh* are recruited in other signalling systems

In *Drosophila*, *wingless* and *hedgehog* are also involved in the patterning of the developing limb. They interact in the same way as in the segments, with *hh* being expressed by cells in the posterior compartment and *wingless* in the nearby anterior cells. The signalling system also uses the other components of the segmentation signalling pathways, such as *patched*. (Phillips, et al., 1990).

In the vertebrates, the *hedgehog* homologue acts as a morphogen in the organisation of pattern in the Zone of Polarizing Activity in the limb, and in the patterning of the neural tube (Echelard, et al., 1993; Krauss et al., 1993; Riddle, et al., 1993).

1.2. The patched (ptc) protein

The *ptc* protein is a transmembrane protein, with no obvious homology to any other known protein. Its transcript is 5.8 kb long, and expressed during most of embryogenesis (Nakano, et al., 1989).

The pattern of expression of *ptc* in *Drosophila* is more dynamic than that of *en* and *wg*. The first expression (transcript) is observed at stage 5, in a mostly uniform fashion (as opposed to the restricted domains of expression of *en* and *wg*). By stage 10, *ptc* is expressed in broad bands in the ectoderm,

complementary to that of *en* but overlapping with *wg*. In stage 12 embryos, the broad ectodermal band is split in two narrow bands, one marking the anterior boundary, one marking the posterior boundary (the *wg*-expressing cells). This pattern persists to the end of embryogenesis.

In *ptc* mutant embryos, the expression of *wg* broadens; this is because *wg* does not depend on *hh* expression anymore (Ingham and Hidalgo, 1993; Ingham, et al., 1991); indeed, *ptc-hh*.double mutant expresses the same phenotype (Hooper, 1994) as *ptc* mutant. This has led to suggestions that *ptc* might be the receptor for *hh*.

The putative hh and wnt signalling pathways in the leech.

There is so far some evidence that some segment polarity genes are expressed in the leech at a time consistent with the establishment of segment borders (Kostriken & Weisblat, 1992; Lans et al., 1993), i.e. during early stage 7 (see Chapter I). The genes that have been cloned, *en* and *wnt* are potential components of the *hh* and *wnt* signalling pathways. But because there is nothing known about the molecules they interact with (downstream target for *en* or downstream response to the *wnt* signal), it is not possible yet to draw a complete picture of what the signalling pathway may be like in the leech, or whether it could be involved in one way or another in setting up the posterior border of the segmental body of the embryo. There have also recently been reports that *hh* was present in the leech (Chang, et al., 1994), which suggests that the *hh* and *wnt* signals might be conserved.

Here, I report the expression pattern of a putative *ptc* gene, which could be involved in the transduction pathway between *en* and *wnt*.

Material and methods

Embryo collection

Theromyson tessulatum and *H. robusta* embryos were used for this experiment. Adult *Theromyson tessulatum* were collected in the wild during spring and kept in the lab at 14°C or room temperature, allowing for embryos to be collected during about 3 months. For *H. robusta,*, see Chapter II for maintenance of the colony. Embryos were collected and staged, left to develop at 23°C in Htr (see Chapter II), and fixed between stages 4 and 8.

Immunolocalisation in embryos

Since no antibody staining to a membrane protein was previously described in *Glossiphoniid* embryos, the fixation technique had to be adapted, partly from the technique described in *Drosophila* for *ptc* antibody staining, and partly from other *Glossiphoniid* protocols for antibody staining (Nardelli-Haefliger et al., 1994). The embryos were fixed in 4% paraformaldehyde in HBS (Hepes Buffered Saline) for 15 minutes only and rinsed in HBS while removing the vitelline membrane manually.

The embryos were then preblocked and permeabilised in PAT (HBS,1% BSA, 0.1% Triton-X100) for 2h at room temperature or overnight at 4°C, in an microfuge tube without rolling (the embryos are too fragile). The embryos were resuspended in fresh PAT, and incubated in a 1/8 dilution of the 5E10 monoclonal antibody (see Result section) in PAT, overnight at 4°C, or 5h at room temperature. After a 10 minutes wash in PAT, I did 3 x30 minute washes in PBT (HBS, 0.1% BSA, 0.1% Triton-X100) containing 3% Normal Goat Serum. The secondary antibody (Cappel fluorescein-conjugated goat anti-mouse antibody) was used at a dilution of 1/200 for 2h at room temperature or overnight at 4°C. The final washes were: 1x 10 min in PBT and 3x 10 min in PTW (HBS, 0.1% Tween 20).

Viewing the results

The embryos were counterstained with either Hoechst or with Daunomycin (see Chapter II). Both are fluorescent nuclear counterstains. Hoescht was used when viewing the samples under conventional fluorescence (UV channel); Daunomycin was used when viewing the samples on the confocal microscope (BioRad), using the Rhodamine channel.

The embryos were mounted in Glycerol: PBS (4 : 1), 4 % propyl galate, in a window carved in 2 layers of electric tape, between slide and coverslip.

Cloning of patched

RNA extraction

RNA was extracted from *H.robusta* embryos of different stages: synchronous clutches (containing between 10 and 30 embryos) were isolated and ground together in a microfuge tube, adding 600µl of RNA extraction buffer (Sambrook et al., 1989): 50mM NaCl, 50mM trisCl (pH 7.5), 5mM EDTA pH8, 0.5% SDS, 200 µg/ml proteinase K. The extraction was followed by a LiCl precipitation (Sambrook et al., 1989). The RNA was resuspended a final volume of 10 µl. 5µl was run on a gel, and 5µl used for the 1st strand synthesis.

3 RNA extractions were done on 3 different batches of embryos: batch n°1 was 7 clutches of stage 2, 3, and 4 embryos; batch n°2 was 7 clutches of stage 7 embryos; batch n°3 was 12 clutches of stage 8 embryos.

First strand cDNA synthesis

I used the Boeringer 1st strand kit (5µl RNA, 3µl H_2O). The RNA was denatured at 70°C for 10 min, then kept on ice. To the RNA was added 5µl bulk buffer, 1µl DTT, 1µl $pd(N)_6$ (random hexamer primers). cDNA was

synthesed at 37°C for 1h. 2µl were checked on an agarose gel, and the other 18µl were kept at -20°C for further use in the PCR.

PCR

PCR was done using two different sets of primers, the *Rev* (*R*) and the *Genie* (*G*) primers (see Figure V.2). Different conditions were used for the two sets, as described below. When changes were made in these conditions, it is indicated in the appropriate Results section.

For the Rev primers (R2 and R4), in a 50µl reaction, I used:

5µl PCR buffer (10x), for a final MgCl concentration of 1.5mM

0.5 µl dNTP [25mM], for a final concentration of 250µM

1µl R_2 (250 ng) for a final concentration of 0.5µM

1µl R4 (250 ng) for afinal concentration of 0.5µM

2µl sample (cDNA)

0.2µl Taq polymerase

The temperature conditions were: hot start at 94°C for 5'; holding temperature 72°C while the enzyme is being put in; then, 30 cycles of: 94°C for 30" ; 50°C for 30" ; 72°C for 90". In the final cycle, the polymerising temperature is held for 5'.

For the Genie primers: the Magnesium concentration was increased (decreases stringency) as well as the concentration of primers. In a 50µl reaction, I used:

5µl PCR buffer (10x)

2µl $MgCl_2$ (25mM) for a final total concentration of 2.5mM

0.5 µl dNTP [25mM], for a final concentration of 250µM

4µl G_1 (1µg) for a final concentration of 2µM

4µl G_2 (1µg) for a final concentration of 2µM

2µl sample (cDNA)

0.2µl Taq polymerase

The temperature conditions were the same as for the Rev primers.

Figure V.2

(A)

```
   1  MDRDSLPRVP DTHGDVVDEK LFSDLYIRTS WVDAQVALDQ IDKGKARGSR   50
  51  TAIYLRSVFQ SHLETLGSSV QKHAGKVLFV AILVLSTFCV GLKSAQIHSK  100
 101  VHQLWIQEGG RLEAELAYTQ KTIGEDESAT HQLLIQTTHD PNASVLHPQA  150
 151  LLAHLEVLVK ATAVKVHLYD TEWGLRDMCN MPSTPSFEGI YYIEQILRHL  200
                 Rev4 →
 201  IPCSIITPLD CFWEGSQLLG PESAVVIPGL NQRLLWTTLN PASVMQYMKQ  250
 251  KMSEEKISFD FETVEQYMKR AAIGSGYMEK PCLNPLNPNC PDTAPNKNST  300
                      Rev2 ←
 301  QPPDVGAILS GGCYGYAAKH MHWPEELIVG GRKRNRSGHL RKAQALQSVV  350
 351  QLMTEKEMYD QWQDNYKVHH LGWTQEKAAE VLNAWQRNFS REVEQLLRKQ  400
 401  SRIATNYDIY VFSSAALDDI LAKFSHPSAL SIVIGVAVTV LYAFCTLLRW  450
 451  RDPVRGQSSV GVAGVLLMCF STAAGLGLSA LLGIVFNAAS TQVVPFLALG  500
 501  LGVDHIFMLT AAYAESNRRE QTKLILKKVG PSILFSACST AGSFFAAAFI  550
 551  PVPALKVFCL QAAIVMCSNL AAALLVFPAM ISLDLRRRTA GRADIFCCCF  600
 601  PVWKEQPKVA PPVLPLNNNN GRGARHPKSC NNNRVPLPAQ NPLLEQRADI  650
 651  PGSSHSLASF SLATFAFQHY TPFLMRSWVK FLTVMGFLAA LISSLYASTR  700
 701  LQDGLDIIDL VPKDSNEHKF LDAQTRLFGF YSMYAVTQGN FEYPTQQQLL  750
 751  RDYHDSFVRV PHVIKNDNGG LPDFWLLLFS EWLGNLQKIF DEEYRDGRLT  800
                                          Genie1 →
 801  KECWFPNASS DAILAYKLIV QTGHVDNPVD KELVLTNRLV NSDGIINQRA  850
 851  FYNYLSAWAT NDVFAYGASQ GKLYPEPRQY FHQPNEYDLK IPKSLPLVYA  900
                                    Genie2 ←
 901  QMPFYLHGLT DTSQIKTLIG HIRDLSVKYE GFGLPNYPSG IPFIFWEQYM  950
 951  TLRSSLAMIL ACVLLAALVL VSLLLLSVWA AVLVILSVLA SLAQIFGAMT 1000
1001  LLGIKLSAIP AVILILSVGM MLCFNVLISL GFMTSVGNRQ RRVQLSMQMS 1050
1051  LGPLVHGMLT SGVAVFMLST SPFEFVIRHF CWLLLVVLCV GACNSLLVFP 1100
1101  ILLSMVGPEA ELVPLEHPDR ISTPSPLPVR SSKRSGKSYV VQGSRSSRGS 1150
1151  CQKSHHHHHK DLNDPSLTTI TEEPQSWKSS NSSIQMPNDW TYQPREQRPA 1200
1201  SYAAPPPAYH KAAAQQHHQH QGPPTTPPPP FPTAYPPELQ SIVVQPEVTV 1250
1251  ETTHSDSNTT KVTATANIKV ELAMPGRAVR SYNFTS
```

(B)

Primer REV4

GGACGAATTC YTN GAY TGY TTY TGG GA
L D C F W

Primer REV2

GGACGAATTC T(C/G) YTC N(T/G)G CCA RTG CAT
E/Q E P/Q W H M

Primer GENIE1

GGACGAATTC GAY GGN ATH ATH AAY C
D G I I N Q/P

Primer GENIE2

GGACGAATTC RTA YTG RTC CCA RAA NA
Y Q E W F I/L

SYMBOLS FOR MIXED MERS

B = (C,G,T) R = (A,G)
D = (A,G,T) S = (C,G)
H = (A,C,T) V = (A,C,G)
K = (G,T) Y = (C,T)
M = (A,C) W = (A,T)
N = (A,C,G,T)

Figure V.2 The degenerate primers used for the *Rev* and the *Genie* PCR reactions. **(A)** *D.melanogaster* sequence of *ptc* with localisation of the primers used. C terminal of the protein is at the top. **(B)** sequence of the primers. All DNA sequences are 5' to 3'. Below is the amino acid translation where appropriate.

PCR were performed on a Perkin Elmer Cetus machine except in the second attempt at the Rev PCR, where a Hybaid machine was used.

Cloning

PCR reactions were run on agarose gels (1 to 1.2%) and the interesting fragment was purified (see Chapter IV). Cloning was either done as for *hairy* (Chapter IV), or cloned into EcoRI cut pBsk. In that case, the fragment was EcoRI digested, phenol chloroform extracted, precipitated, and resuspended in 20µl before ligation into the vector.

Ligation was done in 25µl, using Promega ligase and buffer, 20µl fragment and 1µl pBsk vector (100 ng/µl), at 15°C overnight.

E. coli were transformed as in Chapter IV, and the colonies screened for the presence of an insert by miniprep digest, using EcoRI for the fragments cloned in pBsk, and PstI and SphI for the fragments cloned in pGem (see Chapter IV).

Sequencing

Sequencing was done as in Chapter IV.

Sequence comparison

The sequences were first entered on the Genejockey software, virtually translated, and compared by eye with the *Drosophila*, and zebrafish sequences. Hydrophobicity plots generated by the software were also compared. Sequences were then entered on Genebank and the FASTA program was used to find sequence similarities.

Results

The patched (ptc) pattern of expression: analysis of the Mab 5E10 antigen distribution.

A preliminary study was conducted on the *ptc* gene using a monoclonal antibody raised against the *Drosophila ptc*, Mab5E10. The

antibody was raised against a fusion protein containing the N-teminal section of the Drosophila *ptc* (Wendy Norris, personnal communication).

On the basis of a possible cross-species reaction, I used the Mab5E10 to immuno-stain different stages and different species of leeches to increase the probability of a cross-reaction. I used fluorescent-conjugated antibody and the labelled embryos were examined by confocal microscopy. The 5E10 antibody gave more definite results in *T. tessulatum* than in *H. robusta* and most of the results described are from my observations on *T. tessulatum.*

Experimental staining was variable among embryos of the same batch, and between batches. Control immuno-localisation experiments in which either the Mab5E10 or the secondary antibody were used alone were devoid of signal (Fig. 4 (A), and Fig. 5 (A)). A total of 11 experimental batches of approximately 6 embryos between stage 4 and 8 were examined. The strongest signal came from the periphery of the cells, in patches, consistent with the idea that Mab5E10 recognises a membrane protein. However, some embryos (from 0 embryos in 4 batches to all embryos in 3 batches) at all stages also presented a signal in the cytoplasm and in the nucleus. This was more difficult to understand considering the nature of the antigen Mab5E10 was supposed to recognise. This staining sometimes appeared in embryos (at least 2) where membrane signal was detected, or sometimes in embryos (at least 2) with no membrane signal. Because the nuclear signal did not always follow the same pattern as the membrane signal, both are described independently. Each of the patterns described below were observed on at least 2 embryos com ing from different layings and different staining batches.

Mab5E10 signal is first detected at around stage 6 (Fig. V. 3 and V. 4) in the micromere cap (precursor of the provisional epithelium), and in the anterior part of the embryo, possibly in the micromere-derived cells that will give rise to the proboscis (unsegmented part of the head). The signal

Figure V. 3. Fluorescent immunostaining of wholemount*Theromyzon tessulatum* at stage 6: the peripheral signal. (A, B, C, D) confocal imaging, (E, F, G, H), line drawings of the confocal images to outline the morphology of the embryos. (A,E) Side view onto the micromeres. The signal is strong in all the micromeres, but the nuclei do not appear. (B, F), Top (dorsal) view on the micromeres. The signal in the micromeres is in the periphery of the cells. (C,G) Top view on the micromeres. The embryo is damaged, but the signal very strongly outlines 2 cells, presumably micromeres, underneath the macromeres. (D,H) Top view on the micromeres. The signal outlines the macromeres (see between *A*, *B* and *C*), but more strongly the micromeres.
Unless other wise stated, in E, F, G,and H, the thin lines represent the outline of the micromeres (*m*); *A*, *B* and *C*: macromeres; *T*: teloblasts.

Figure V. 4. Fluorescent immunostaining of wholemount *Theromyzon tessulatum* at stage 6. The nuclear signal, except (B, F), peripheral signal. (A, B, C, D) confocal imaging, (E, F, G, H), line drawings of the confocal images to outline the morphology of the embryos. (A, E) Control, stage 6b, dorsal, slightly posterior view; the micromers are on the dorsal side (up). The teloblasts and the micromers are outlined due to the autofluorescence of the yolk. (B, F) ventro-posterior view (the micromers are out of the field of view). On the left, the left M bandlet is visible. The staining is localised between the teloblasts and between the teloblasts and the macromers. The nuclei of the M bandlet are not stained. (C, G) Stage 6b, dorsal view. No patchy membrane staining is visible but the nuclei of the micromeres and of the M bandlet are immuno-stained. (D, H) Stage 6, dorsal view, squashed. Only the micromers (no bandlet) are visible. The nuclei are stained. Some patchy signal is observed but it is not above the level observed in some controls.
Scale bar: approximately 100µ. All pictures were taken at magnification x50 with a water immersion lens.
A, B, C: macromers A, B and C; *N, OP, Q*: teloblasts; *T*: undefined teloblast; *m*: micromers; *b*: bandlet.

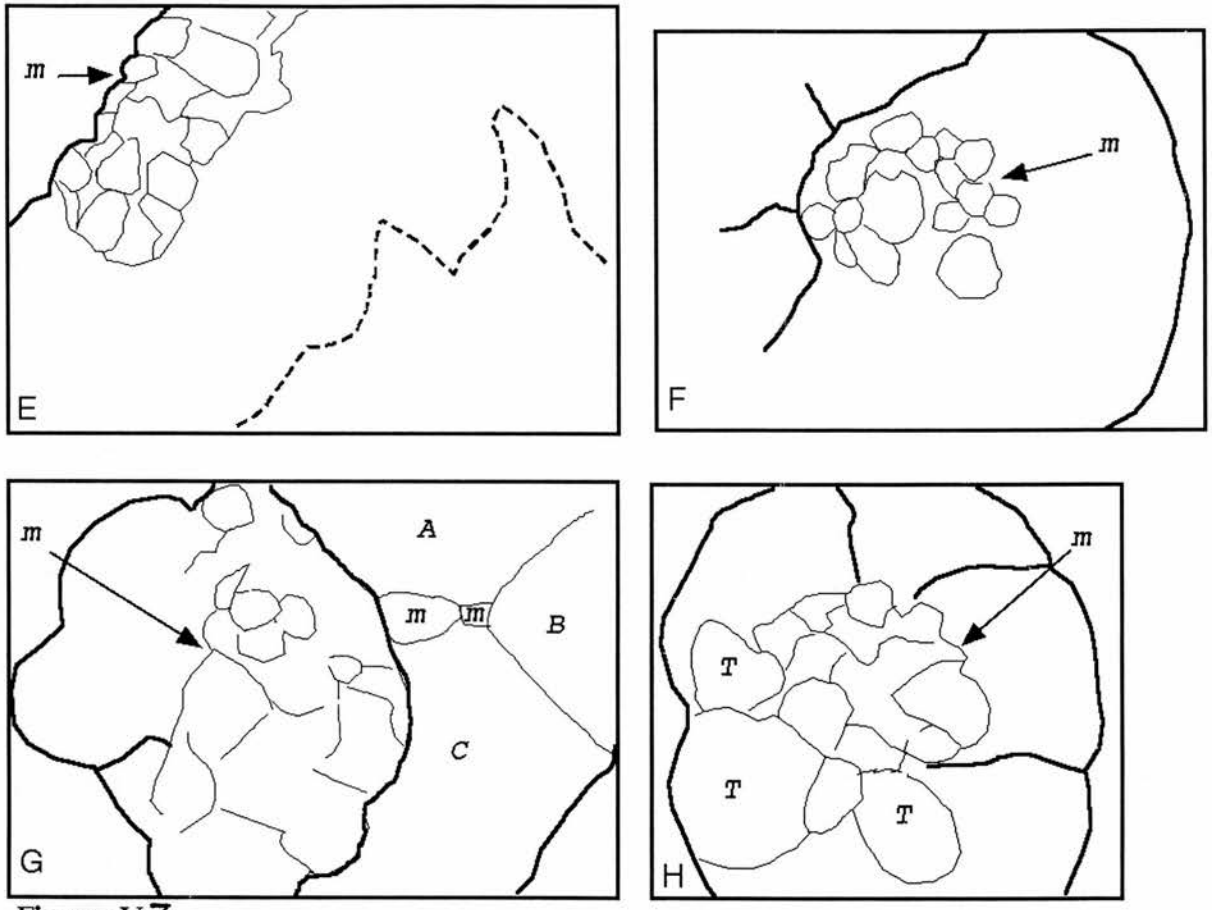

Figure V.3

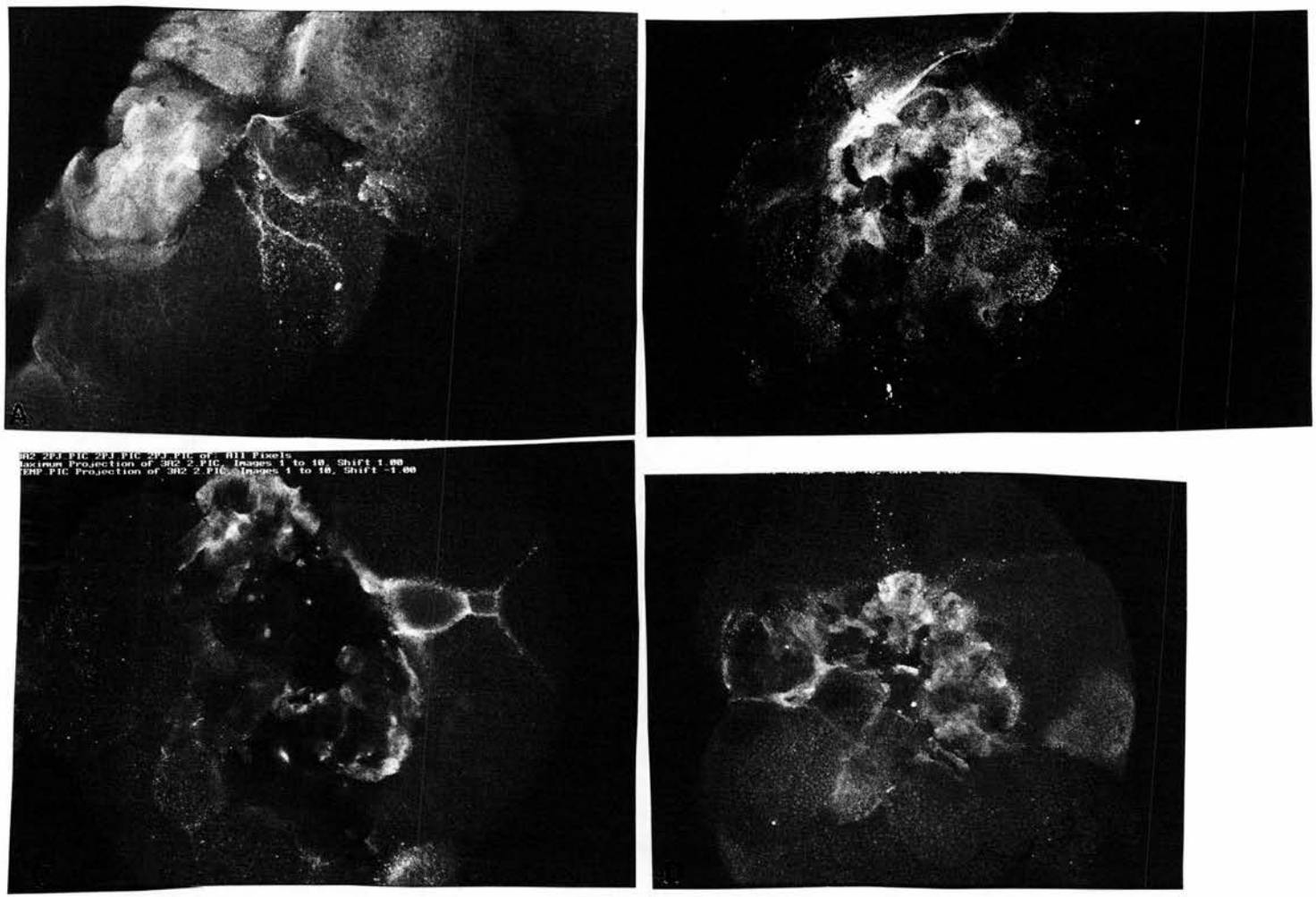
A
Maximum Projection of 3R2 2.PIC, Images 1 to 10, Shift 1.00
TEMP.PIC Projection of 3R2 2.PIC, Images 1 to 10, Shift -1.00

Figure IV.3

Figure V. 4. Fluorescent immunostaining of wholemount*Theromyzon tessulatum* at stage 6. The nuclear signal, except (B, F), peripheral signal. (A, B, C, D) confocal imaging, (E, F, G, H), line drawings of the confocal images to outline the morphology of the embryos. (A, E) Control, stage 6b, dorsal, slightly posterior view; the micromeres are on the dorsal side (up). The teloblasts and the micromeres are outlined due to the autofluorescence of the yolk. (B, F) ventro-posterior view (the micromeres are out of the field of view). On the left, the left M bandlet is visible. The staining is localised between the teloblasts and between the teloblasts and the macromeres. The nuclei of the M bandlet are not stained. (C, G) Stage 6b, dorsal view. No patchy membrane staining is visible but the nuclei of the micromeres and of the M bandlet are immuno-stained. (D, H) Stage 6, dorsal view, squashed. Only the micromeres (no bandlet) are visible. The nuclei are stained. Some patchy signal is observed but it is not above the level observed in some controls. Scale bar: approximately 100µ. All pictures were taken at magnification x50 with a water immersion lens.
A, B, C: macromeres A, B and C; *N, OP, Q*: teloblasts; *T*: undefined teloblast; *m*: micromeres; *b*: bandlet.

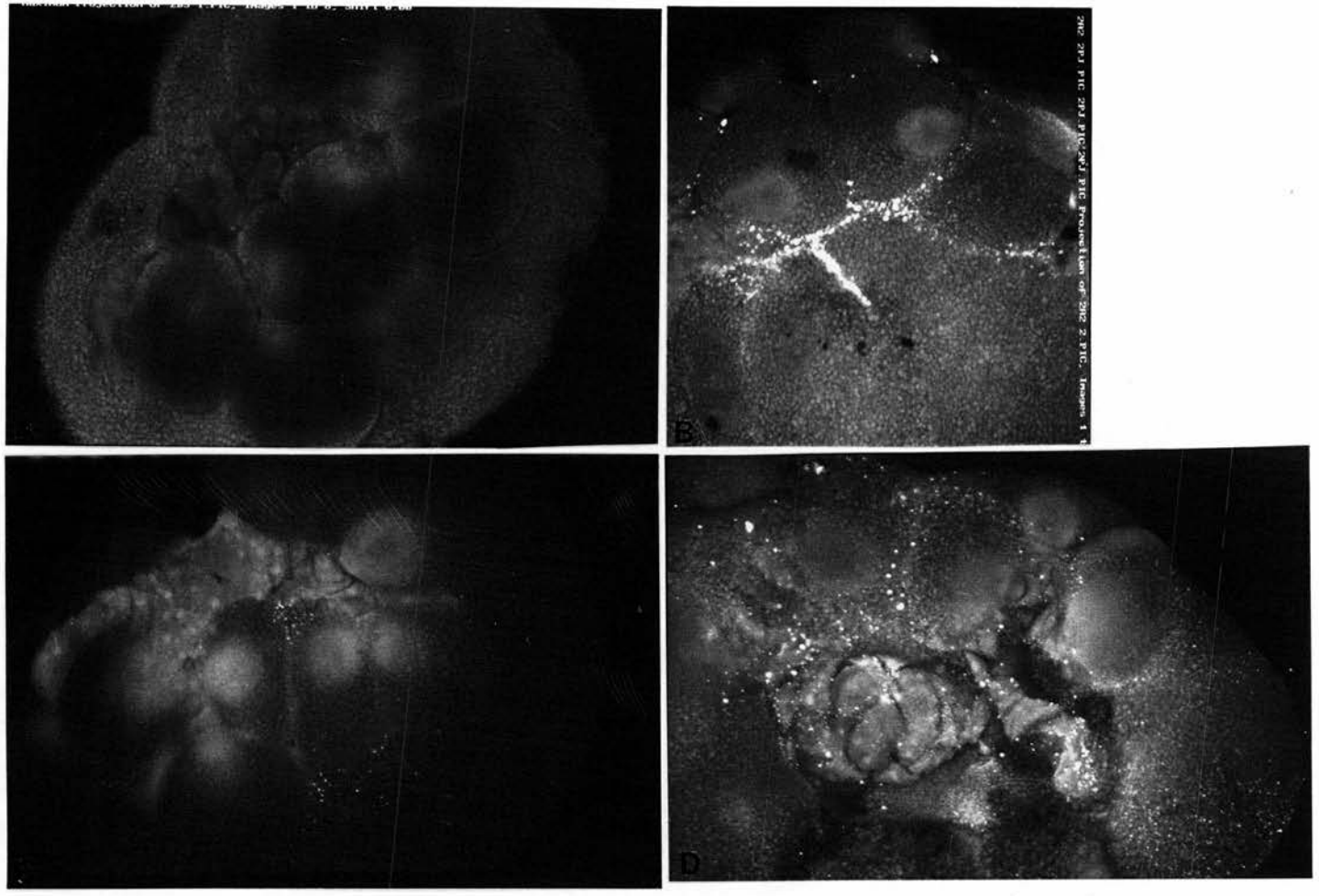

Figure V. 4

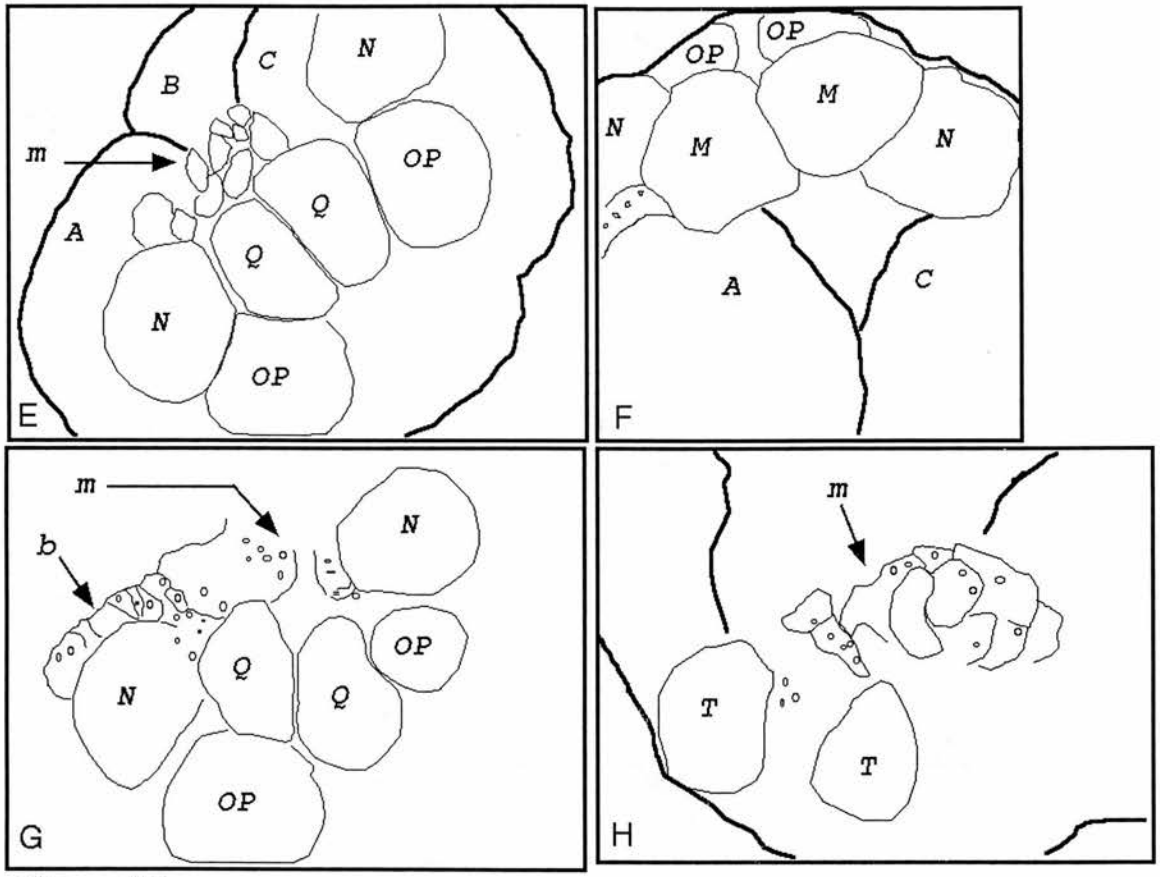

Figure V.4

Figure V.5. Fluorescence immuno-staining of wholemount *Theromyzon tessulatum* at stage 8. (A, B, C, D) confocal imaging, (E, F, G, H) line drawing of the confocal images to outline the morphology of the embryos. Most immunological signal has disappeared, although in the bandlet, sometimes, the staining is clearly above the control. Anterior is up. (A, E) control (no primary antibody), late stage 8. Ventral view.the large fluorescent dots were sometimes observed, all over the embryo (yolk as well as cells). The staining was neither patchy nor in the nuclei. (B, F) early stage 8, dorsal view. The staining is patchy and peripheral. The bandlets have the highest intensity of staining at the posterior end (near the teloblasts). (C, G) late stage 8, ventral view. The bandlets in the germinal band are outlined by the fluorescent signal. The nuclei of the germinal band and the germinal plate are also highlighted (white arrows). (D,H) Middle stage 8, dorso-posterior view (Anterior is at the top, dissappearing out of the field of vision). The nuclei of the bandlets are very visible, as well as the nuclei of the expanding micromere cap (white arrows)
GB: germinal band; GP: geminal plate; B: bandlet; T: teloblast; M: midline; MC: micromere cap.

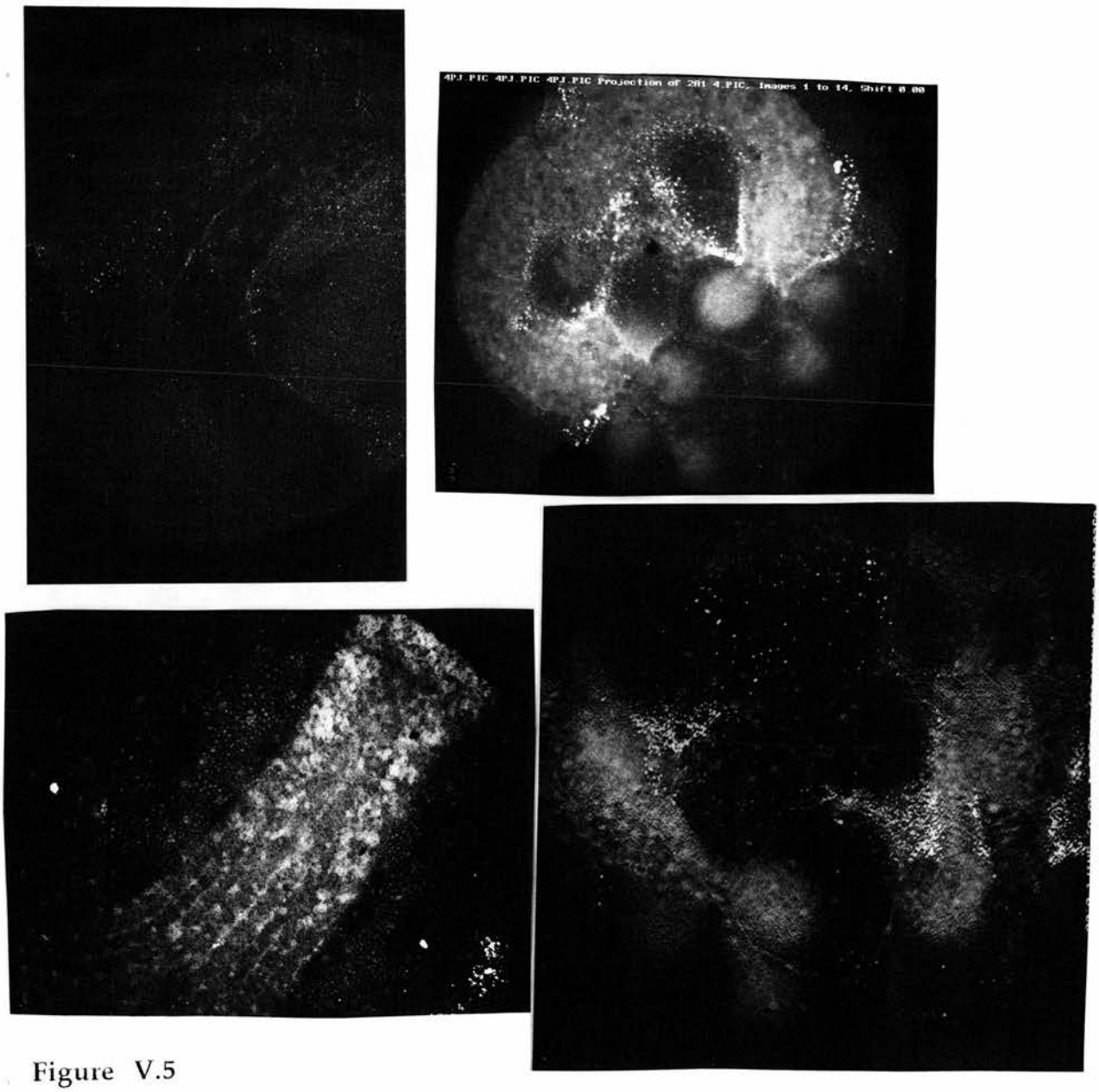
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Figure V.5

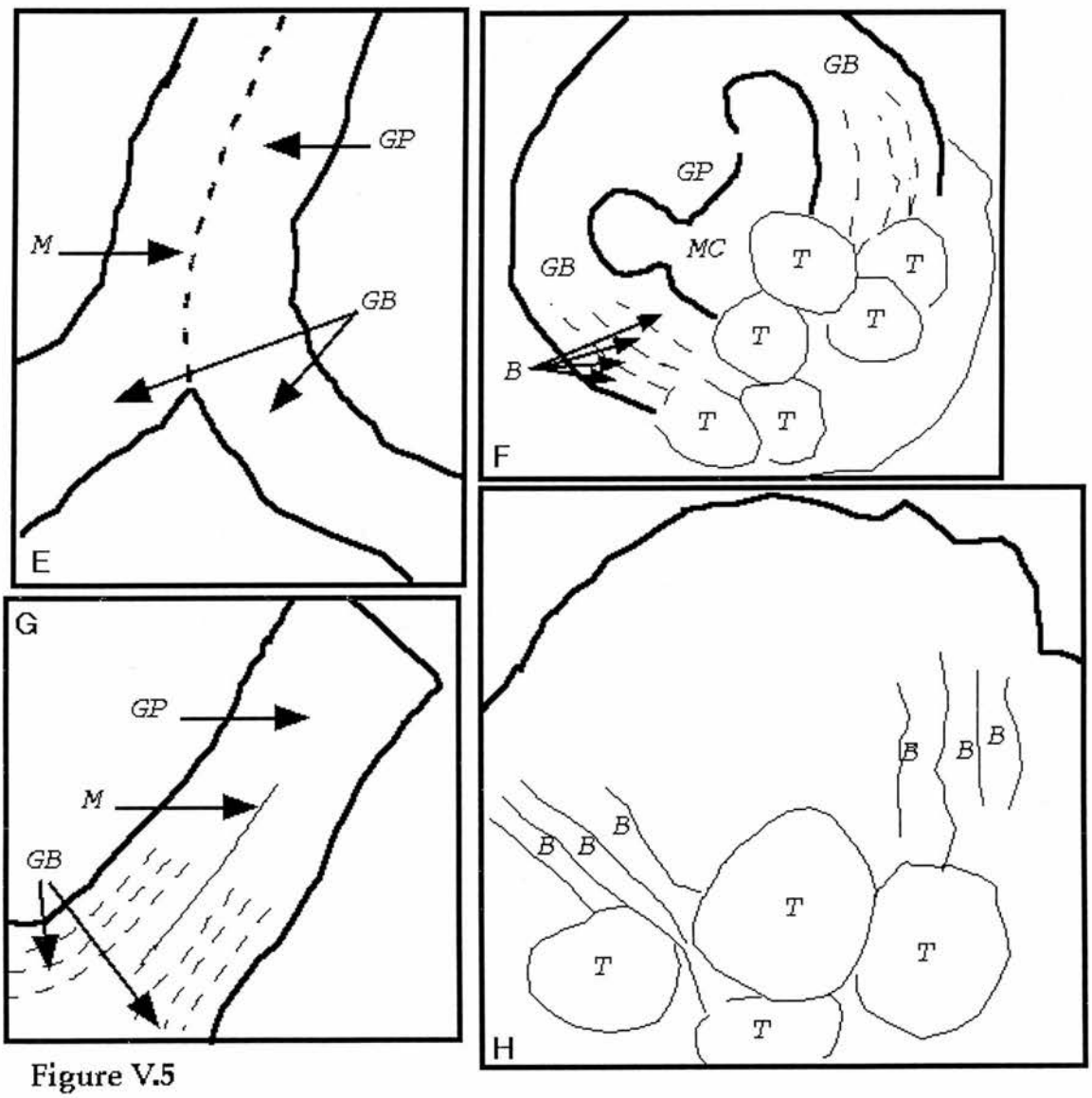


Figure V.5

was the strongest in the provisional epithelium but it is not possible to tell which cells exactly (descendent of which micromeres) express the Mab5E10 antigen without the help of lineage tracer (Kostriken & Weisblat, 1992). In some of the embryos, nuclear staining was also observed in the most anterior part of the left M bandlet (See Figure V.4).

After stage 6, the staining dis appeared from the micromere cap in all the embryos observed. The staining was less consistent in later stage 8 (see Figure V.5) with a strong variation between embryos of the same batch. Sometimes, the staining detected was at the level of the background, sometime there was some staining in the germinal bands, between the bandlets and in some of the teloblasts. At early stage 8, the staining in the germinal bands was present uniformly. The signal between the different ectodermal bandlets was of similar intensity (see Fig. V. 5C), but was not compared to the mesodermal bandlet.

At later stages of development (late stage 8), the antero-posterior gradient of intensity of the signal is not so strong (see Fig. V. 5C). The signal was still visible around the teloblasts and at the lateral border of the bandlets (see Fig. V.5D). Although this staining was strongest in the membranes, it was also detected in the nuclei (see Fig. V.5D). However, the staining detected in the nuclei was more ubiquitous, since it was also detected in the nuclei of the provisional epithelium (compare Figure V.5 with Figure I .2B of a general nuclear staining of a similar stage embryo).

Due to the small number of embryos observed and the high variability of the pattern, no definite conclusion can be drawn until further stainings are observed, and quantitation are done. This has not been possible so far due to the combined facts that *T. tessulatum* is a seasonal leech and no more Mab5E10 is currently available.

The cloning of the patched homologue in the leech

RNA was extracted from embryos in batches according to their stage (see Chapter I for embryonic stages): batch 1 contained early stages (2 to 4), batch 2 intermediate stages (stage 7), and batch 3 late stage (stage 8) (see Figure V.6). The RNA was reverse transcribed into cDNA (see Figure V.6).

The Rev PCR

PCR on the cDNA from batches 1, 2 and 3 amplified fragments of the expected size (see Figure V.7) around 350bp, as well as other, larger fragments. The 350 bp fragment from the PCR on batch 2 was cloned. The band was purified, cloned into pBsk and 2 clones sequenced (see Figure V.8A: R5 and R8). These sequences showed no homology either to the *Drosophila* or the zebrafish *patched* gene outside the primer sequence.

Because in the first attempt the number of clones was not large enough, another PCR was run in a second attempt. The second PCR was run using the product of the first PCR from batch 2 as a template. This yielded clones which were sequenced, were different from the clones obtained in the first PCR, but again did not resemble *patched* from other species (see figure V.8: R16). This could be explained by the fact that in both cases only a small number of clones (2 or 3) were sequenced. It is probable that in both cases the population of DNA fragment obtained in the PCR was mixed, and the clones sequenced might not reflect the total population of DNA fragments.

The Genie PCR

The Genie PCR was not as straightforward and needed to be optimised before any fragment could be cloned. Different concentrations of $MgCl_2$ were used from 2.5mM to 5mM, resulting in an increase in the DNA amplified. However, amplification at high concentration of $MgCl_2$ resulted in a smear, due to the decrease of binding specificity of the primers. To counterbalance the lowering of stringency induced by the high

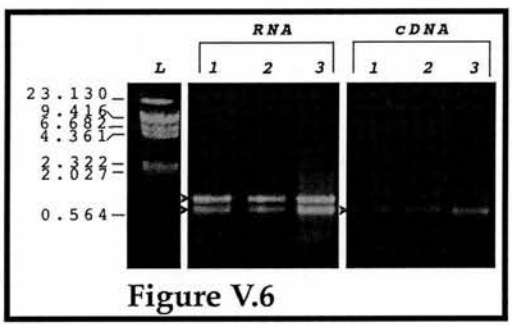


Figure V.6

Figure V.6. Total RNA extraction from batches 1 (early stages), 2 (intermediate stages) and 3 (late stages). The two bands (arrowheads) are the ribosomal RNA, much in excess to the mRNA in the cells. This shows that that there has been little degradation. The fact that there is no signal in the high molecular weight also shows that the DNA contamination is minimal (if not inexistant). In the first strand cDNA synthesis, the smearing indicates that cDNAs of different lengths have been synthesised. However, there is still a high level of ribosomal RNA (arrowhead). The size markers are Lambda DNA markers for both RNA and cDNA, The sizes are expressed in kb.

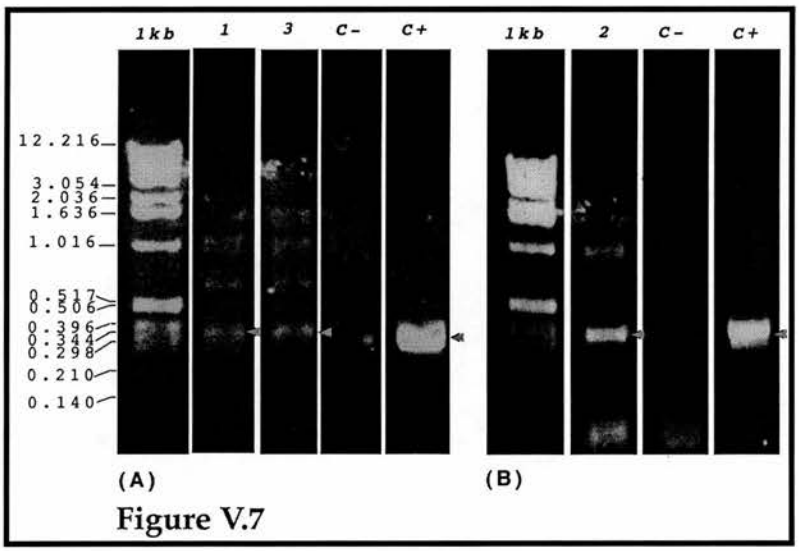


Figure V.7

Figure V.7 Rev PCR on batches 1, 2 and 3. Batch 2 PCR (B) was run independently from 1 and 3 (A). In both cases, a negative control (C-) was run, with water instead of DNA in the reaction. In both cases, no band appeared. In both cases, a positive control (C+) was run, using a *Drosophila patched* clone. In both cases, a signal appeared around 350bp as expected.

In the 3 batches (1, 2, and 3), a bright band appeared around 350 bp (arrowheads) as in the *Drosophila* controls (C+). The fragment in the PCR 2 was purified and cloned. Other, lighter bands appeared, reflecting the fact that the primers are degenerate, and therefore might amplify unspecific DNA or a different *patched* gene. At this point it is impossible to tell whether these amplified fragments are related to *patched* or not.

R5

```
      5'TTGGATTGCTTTTGGGAACCGAGTGTCCGTCTGGAGTGGGAGTCGACTCTCGAAACGACA
      1 ---------+---------+---------+---------+---------+---------+ 60
      3'AACCTAACGAAAACCCTTGGCTCACAGGCAGACCTCACCCTCAGCTGAGAGCTTTGCTGT

a     L  D  C  F  W  E  P  S  V  R  L  E  W  E  S  T  L  E  T  T  -
b      W  I  A  F  G  N  R  V  S  V  W  S  G  S  R  L  S  K  R  Q  -
c       G  L  L  L  G  T  E  C  P  S  G  V  G  V  D  S  R  N  D  R -


      GAAGTCATTGAATGGCTGTCTTCAGATACACATATCACTTGCCAGGTACTGAAGCGTGTG
   61 ---------+---------+---------+---------+---------+---------+120
      CTTCAGTAACTTACCGACAGAAGTCTATGTGTATAGTGAACGGTCCATGACTTCGCACAC

a     E  V  I  E  W  L  S  S  D  T  H  I  T  C  Q  V  L  K  R  V  -
b      K  S  L  N  G  C  L  Q  I  H  I  S  L  A  R  Y  *  S  V  C  -
c       S  H  *  M  A  V  F  R  Y  T  Y  H  L  P  G  T  E  A  C  V -


      TGGCCCCCCGCTCAGAGGGACTCGATGTTT3'
  121 ---------+---------+---------+ 150
      ACCGGGGGGCGAGTCTCCCTGAGCTACAAA5'

a     W  P  P  A  Q  R  D  S  M  F  -
b      G  P  P  L  R  G  T  R  C  -
c       A  P  R  S  E  G  L  D  V  -
```

Figure V.8 Sequences of 3 clones obtained by Rev RT-PCR on RNA from stage 8 leech embryo. The sequence obtained and their complementary strands are on the first two lines, followed by virtual translation into amino acid: a, b, and c are the forward translation, d, e and f the translation of the complementary strand. Translations of both strands are provided when the primer was not present (and therefore the coding strand could not be identified). None of these sequences had any homology to the *Drosophila* or zebrafish *ptc* gene outside the primer sequence. The primer sequence, when present, is highlighted in bold.

Figure V.8

R9

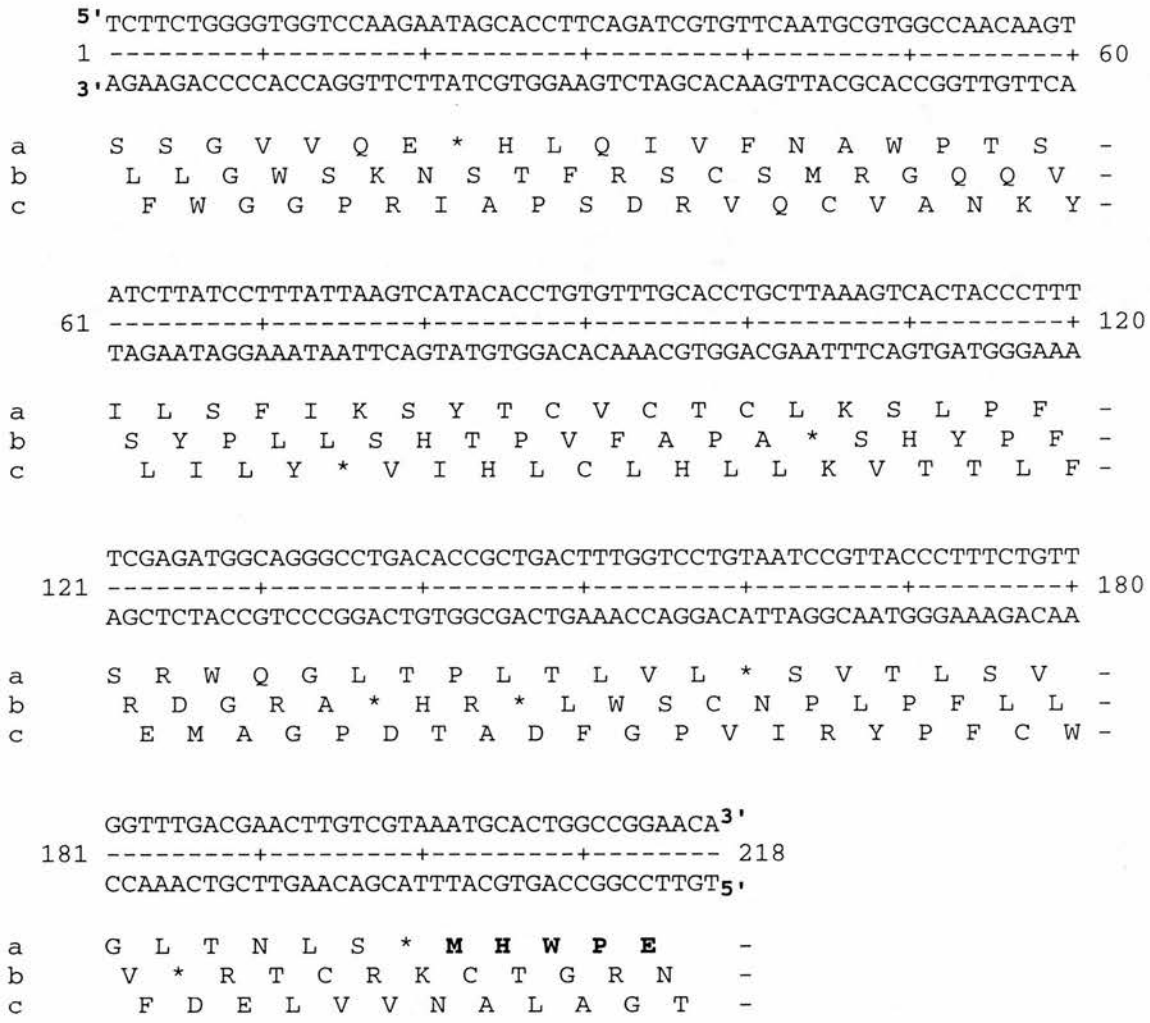
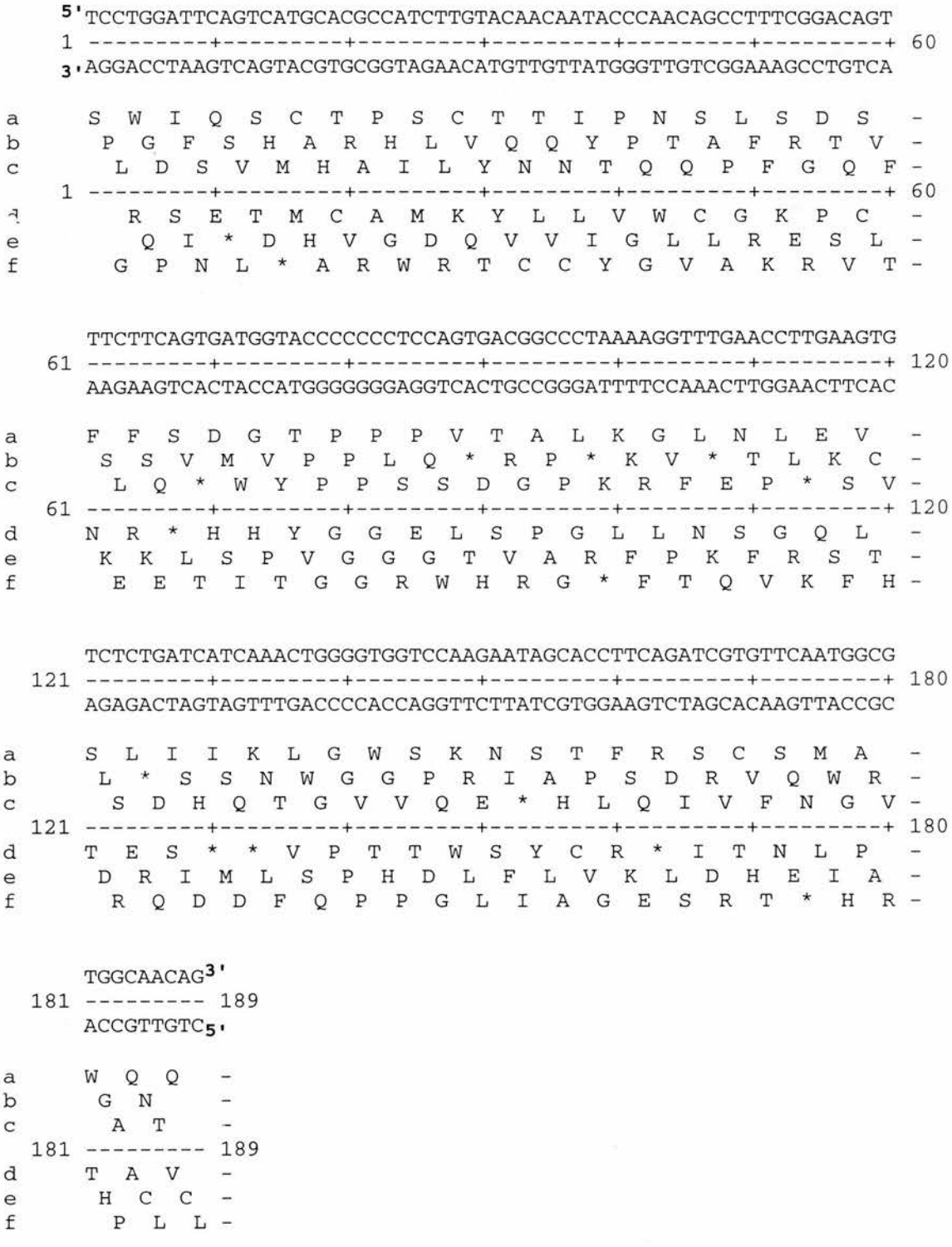

5'TCTTCTGGGGTGGTCCAAGAATAGCACCTTCAGATCGTGTTCAATGCGTGGCCAACAAGT
1
60
3'AGAAGACCCCACCAGGTTCTTATCGTGGAAGTCTAGCACAAGTTACGCACCGGTTGTTCA
a S S G V V Q E * H L Q I V F N A W P T S -
b L L G W S K N S T F R S C S M R G Q Q V -
c F W G G P R I A P S D R V Q C V A N K Y -
ATCTTATCCTTTATTAAGTCATACACCTGTGTTTGCACCTGCTTAAAGTCACTACCCTTT
61
120
TAGAATAGGAAATAATTCAGTATGTGGACACAAACGTGGACGAATTTCAGTGATGGGAAA
a I L S F I K S Y T C V C T C L K S L P F -
b S Y P L L S H T P V F A P A * S H Y P F -
c L I L Y * V I H L C L H L L K V T T L F -
TCGAGATGGCAGGGCCTGACACCGCTGACTTTGGTCCTGTAATCCGTTACCCTTTCTGTT
121
180
AGCTCTACCGTCCCGGACTGTGGCGACTGAAACCAGGACATTAGGCAATGGGAAAGACAA
a S R W Q G L T P L T L V L * S V T L S V -
b R D G R A * H R * L W S C N P L P F L L -
c E M A G P D T A D F G P V I R Y P F C W -
GGTTTGACGAACTTGTCGTAAATGCACTGGCCGGAACA3'
181
218
CCAAACTGCTTGAACAGCATTTACGTGACCGGCCTTGT5'
a G L T N L S * M H W P E -
b V * R T C R K C T G R N -
c F D E L V V N A L A G T -

Figure V.8

R16



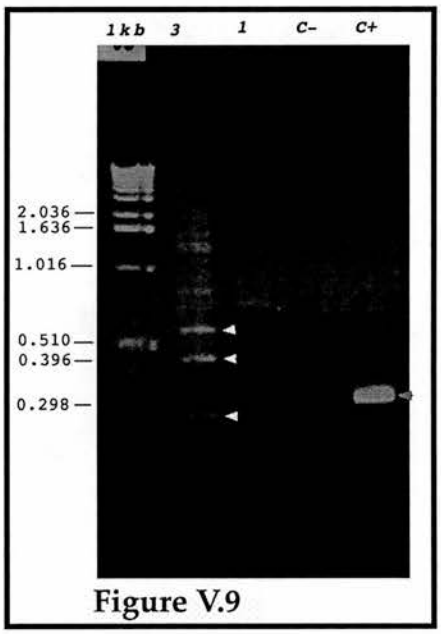


Figure V.9

Figure V.9 Genie PCR on batches 3 and 1. The negative control (water instead of DNA) showed no signal and the positive control (Drosophila DNA) showed a band around 300bp as expected.
The fragments that were isolated and cloned are marked with a white arrow: (1) 280 bp, (2), 400bp, and (3) over 510 bp.

G1-1

Figure V.10 Sequences of 3 clones obtained by Genie RT-PCR on RNA from stage 8 leech embryo. The sequence obtained and their complementary strands are on the first two lines, followed by virtual translation into amino acid: a, b, and c are the forward translation, d, e and f the translation of the complementary strand. Translations of both strands are provided when the primer was not present (and therefore the coding strand could not be identified). None of these sequences had any homology to the *Drosophila* or zebrafish *ptc* gene outside the primer sequence. The primer sequence, when present, is highlighted in bold.

Figure V.10

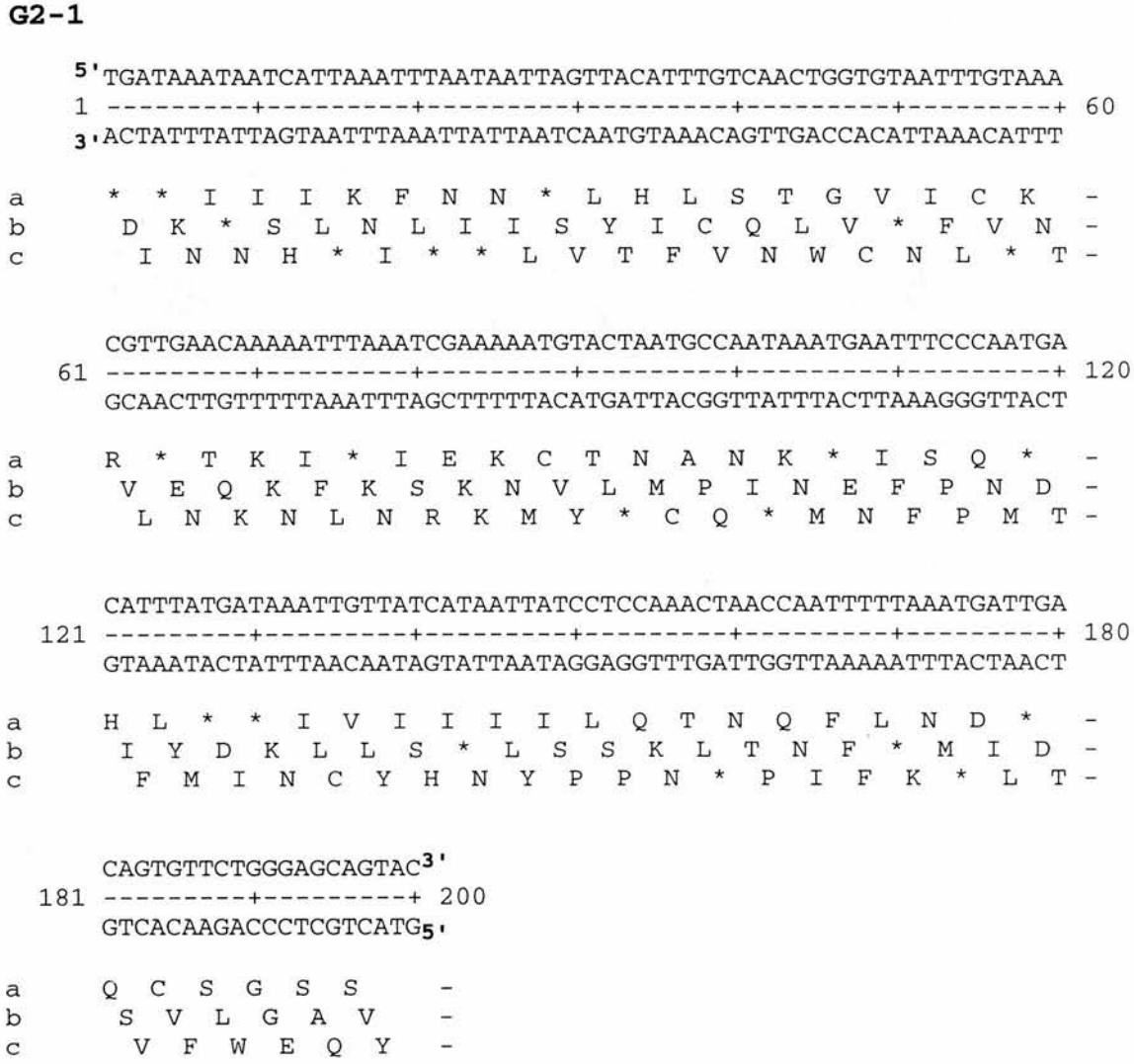

G2-1
5'TGATAAATAATCATTAAATTTAATAATTAGTTACATTTGTCAACTGGTGTAATTTGTAAA
1 ---------+---------+---------+---------+---------+---------+ 60
3'ACTATTTATTAGTAATTTAAATTATTAATCAATGTAAACAGTTGACCACATTAAACATTT
a * * I I I K F N N * L H L S T G V I C K -
b D K * S L N L I I S Y I C Q L V * F V N -
c I N N H * I * * L V T F V N W C N L * T -
CGTTGAACAAAAATTTAAATCGAAAAATGTACTAATGCCAATAAATGAATTTCCCAATGA
61 ---------+---------+---------+---------+---------+---------+ 120
GCAACTTGTTTTTAAATTTAGCTTTTTACATGATTACGGTTATTTACTTAAAGGGTTACT
a R * T K I * I E K C T N A N K * I S Q * -
b V E Q K F K S K N V L M P I N E F P N D -
c L N K N L N R K M Y * C Q * M N F P M T -
CATTTATGATAAATTGTTATCATAATTATCCTCCAAACTAACCAATTTTTAAATGATTGA
121 ---------+---------+---------+---------+---------+---------+ 180
GTAAATACTATTTAACAATAGTATTAATAGGAGGTTTGATTGGTTAAAAATTTACTAACT
a H L * * I V I I I I L Q T N Q F L N D * -
b I Y D K L L S * L S S K L T N F * M I D -
c F M I N C Y H N Y P P N * P I F K * L T -
CAGTGTTCTGGGAGCAGTAC3'
181 ---------+---------+ 200
GTCACAAGACCCTCGTCATG5'
a Q C S G S S -
b S V L G A V -
c V F W E Q Y -

Figure V.10

G2-8

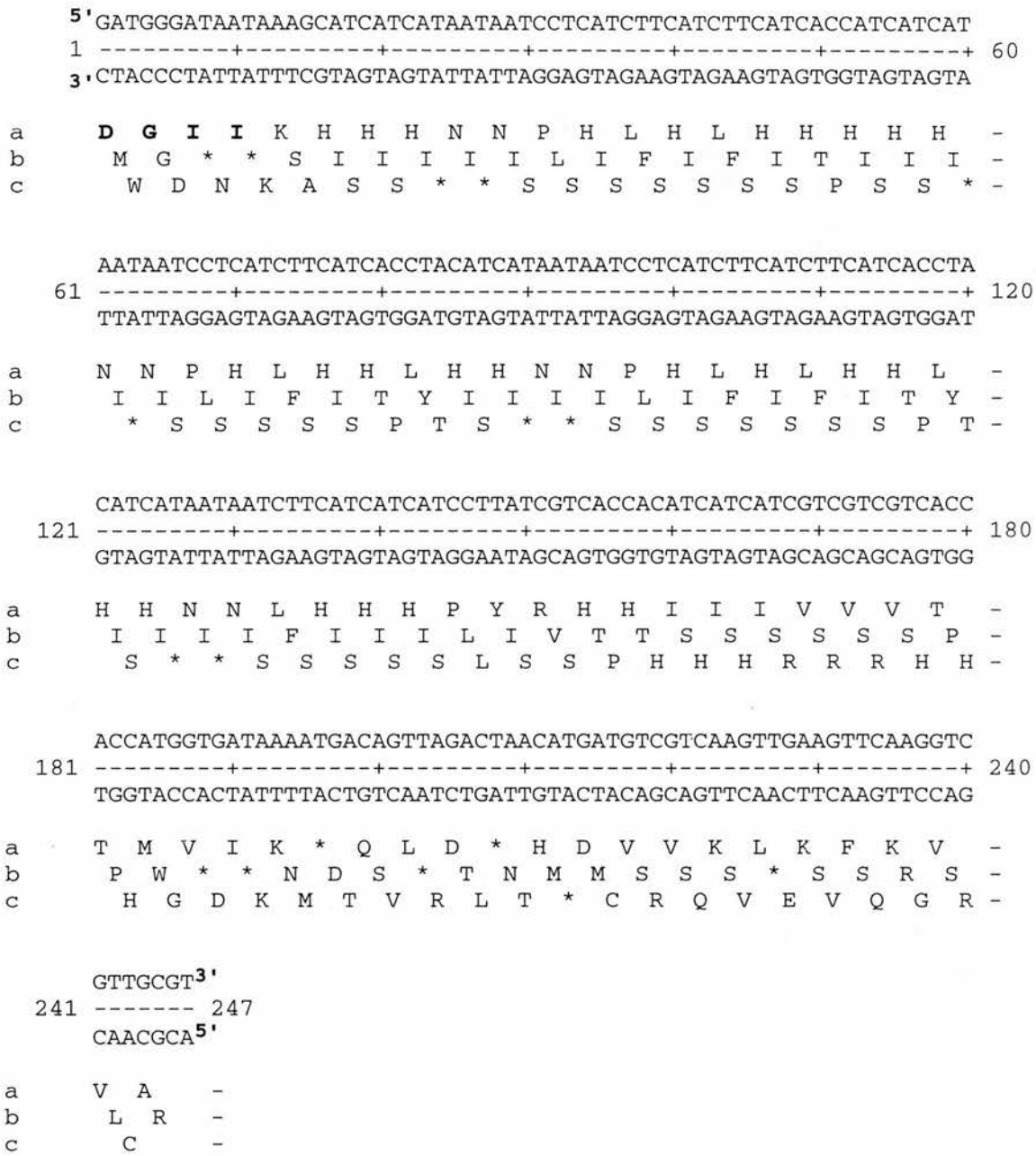
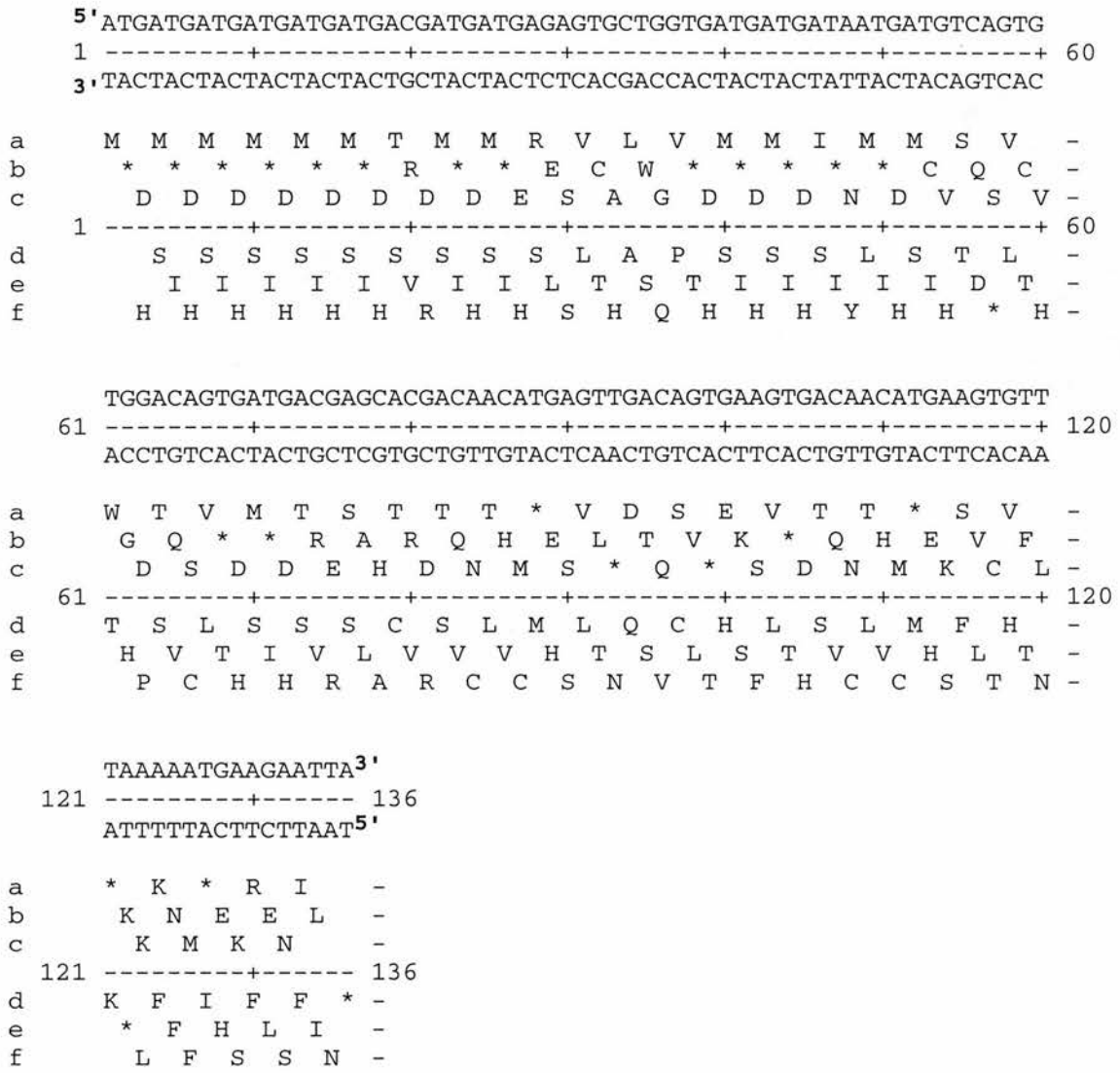


Figure V.10

G2-15

Figure V.10

G2-19



concentration of Mg, the annealing temperature was then increased from 50°C to 55°C resulting in discrete although numerous fragments in the batches 1 and 3 (2 was not used in this reaction) see Figure V.9. Three distinct fragments of approximately 250 bp (a), 400 bp (b) and 550 bp (c) were purified and cloned.

The clones which were sequenced were different from the patched sequences of the other species (Figure V.10).

Discussion

The leech probably has a ptc *homologue.*

Cloning of the ptc gene was unsuccessful, but this could have been due to the fact that the gene is highly divergent from Drosophila.

The fact that the 5E10 antibody cross-reacted in the leech suggests that *ptc* is present. Because the signal appeared in the periphery of the cells, this suggests that the antibody was recognising a membrane-bound protein. However, results from cross-reacting antibodies have to be interpreted with caution, as the antigen recognised in one species may be different from the antigen in another species (Patel et al., 1989; Wedeen and Weisblat, 1991). I also found that the antibody was distributed in the nucleus in some of the cases. This can be interpreted in two different fashions. On the basis of antibody staining in Drosophila, it is more likely that the ptc would have a membrane localisation. It is therefore possible that the 5E10 actually recognises an epitope present on two different antigens in the leech, only one of which would be patched. In the screening of the Mab5E10, the antibody was nover localised in the nucleus (Wendy Norris, personal communication). It is therefore likely that it recognises only one antigen in the Drosophila, but 2 in the leech. Another, less plausible explanation is that the patched protein in the leech may have two different subcellular localisations. This however is not so likely on the basis that (i) the protein is

too large to be anywhere else than in the membrane and (ii) this is not seen in Drosophila, and therefore seems unlikely in the leech.

putative ptc *expression in the micromere cap*

The micromere cap originates from the micromeres born from the macromeres and the teloblast before bandlets start being produced (see Chapter I). During stage 6, the ectoteloblasts (N, O/P, P/O and Q) are still being born and the ectodermal blast cells start being produced by the end of stage 6. The M bandlet starts being formed from the beginning of stage 6, and is visible on the surface of the embryo. At this stage, the micromeres are arranged around the blast cells (Sandig & Dohle, 1988). During stage 7 and 8, the cells of the micromere cap divide and cover the embryo in a movement of epiboly while the segments are being generated (Ho & Weisblat, 1987). The resulting provisional epithelium always covers the ectodermal bandlets where they are joined in the germinal bands.

The putative expression of *ptc* in the micromere cap is therefore too early to have a role in patterning of the segments: the segment precursors (the blast cells) are only starting to be produced. However, it is interesting to note that another segment-polarity homologue gene, *wnt -A* (*wg* homologue) is expressed in the micromere cap at this point (Kostriken & Weisblat, 1992). It is therefore possible that at that stage, *ptc* would be required in the signalling pathway leading to the expression of *wnt-A*, for the patterning within the micromere cap. But, whereas *ptc* expression may be downregulated in the micromere cap after stage 6 or 7, *wnt-A* expression persists in the micromere cap throughout development, and throughout segmentation.

It is difficult to speculate on any possible pathway in the absence of the other protagonists, mostly *hh*. In Drosophila, *ptc* on its own represses *wg*, and *hh* is required to antagonise *ptc* for *wg* to be expressed. *hh* is

reported to be present in the leech (Chang et al., 1994) but its spatio-temporal expression is not yet known.

ptc expression in the germinal band.

After stage 7, the putative expression of membrane-bound *ptc* is restricted to the blast cells and the teloblasts. The intensity was more or less uniform thoughout the germinal band, and no periodic pattern was detected. At this stage, *ptc* is expressed in the same tissue as *en*. The *en* gene expression is itself consistent with a traditional role in segment polarity: it appears in the bandlets as early as stage 7 in the P lineage, at clonal age 36 (Lans et al., 1993). At this stage, the different lineages are still producing blast cells and the different lineages are not yet in register (segment-wise). The different lineages all express *en* in a subset of the progeny of the primary blast cell, but all at a different clonal age, so that the time when the expression is present in the whole segment at once is very short. The expression is therefore very dynamic and it has not yet been possible to compare the expression pattern of *en* and *wnt*. However, if *wnt* and *en* signal to each other for their respective maintenance of expression as in *Drosophila*, we should expect the cells expressing *wnt* and *en* to be very close, since the signal is short range in *Drosophila*. If this was the case, however, *ptc* would be expected to be co-expressed with *wnt* (i.e the micromere cap) and not *en* (see Figure V.1). This is not found during the segmentation stages.

Another hypothesis would be that the *wnt-A* in the micromere cap and the *en* in the germinal band do not signal to each other but are both part of a different signalling pathway: other molecules, such as *decapentaplegic*. could be the downstream target of *ptc* (Capdevila, et al., 1994). In this case, it could be possible that ptc was involved in the reception of a signal (e.g. *hh*) secreted by the *en*-expressing cells.

The study reported here was a preliminary study and because of the high variability of the staining, more staining have to be done in order to draw any conclusion.

Can ptc *have a role in setting up the posterior border?*

From its putative pattern of expression, it seems very unlikely that *ptc* would be involved in the setting up of the posterior border between segmental cells and supernumer ary cells. This would require a more punctual expression only at the site of detachment of the bandlets, which was not found.

Conclusion

Athough no *ptc* gene was isolated from the leech, results from the immunolocalisation using the Mab5E10 support the idea that a *ptc* homologue is present in the leech. Although the distribution of putative *ptc* was variable from one embryo to the next, it was consistently expressed in the micromeres before segmentation (stage 6) and distinctively visible in the bandlets during segmentation (stage 8): the expression of putative *ptc* was dynamic and changed from one tissue to another.

Further hypotheses concerning the role of *ptc* in any signalling would require more information on its potential ligand, *hh*..

Chapter VI. Conclusion

The leech regulates its number of segments through a complex mechanism.

The overall picture that can be drawn from the results presented in this thesis is that the leech regulates its number of segments through a complex mechanism, involving regulation of cell division (of the teloblasts) and specific elimination (of supernumerary cells).

Regulation of the teloblasts' cell cycle

Primarily, a minimum number of cell divisions of each teloblast is required for attaining the proper number of segments (as seen in Chapter II). The M teloblast undergoes up to 41 rounds of cell division, and so produces up to 9 supernumerary cells.

The total number of cell divisions of the teloblasts need not be regulated precisely for the correct number to enter the germinal band. It is possible that the teloblast can divide continuously, but that an external factor prevents it from doing so. The action of such a factor could be time-dependent (since the teloblast always takes exactly the same time to produce the segmental blast cells), or position-dependent.

However, since the number of blast cells born is not constant, it is not from the number of teloblast divisions that the regulation of segment number comes from. It is also unlikely that the teloblasts "count" (assigns a number) to the blast cells as they are born: there is no other system known to count as far as 32.

Establishing the position of the limit between the supernumerary and the segmental cells

Secondarily, the supernumerary cells detach from the bandlet, not all at once but in one lineage after the other. This suggests that the limit

between supernumerary and segmental cells needs to be set in each lineage independently. This does not mean that the posterior border is independent in the different lineages: the position might be concerted to give rise to such a precise border. However, this rules out the possibility that one factor present in the extracellular environment regulates all the lineages simultaneously.

Inducing cell death at the limit between segmental and supernumerary cells

Finally, the detachment of the bandlets happens as a shearing between the segmental cells and the supernumerary cells. It is not clear whether this shearing is the result of the establishment of the posterior border or if the shearing of the cells dictates the position of the border. However, because the anterior supernumerary cells change shape and become elongated, it is more likely that the cells die as a result of the posterior boundary being established.

The more posterior supernumerary cells do not show any sign of death before their elimination. It is even difficult to determine whether these cells really die or are recycled, since they disappear soon after detachment. In any case, their disappearance is a cause rather than a consequence of the trimming of the bandlets.

What the culture says

The culture experiment brought the information that an absence of ectodermal lineages could not be sufficient to decide whether a cell should die or not. In the light of this result, we could suggest that either (i) the cells are born with an identity, which makes them independent of neighbours (the more anterior segmental cells) or dependent on neighbours (the cells at the border between segmental and supernumerary) or (ii) some other part of the environment (not present in the culture) is responsible for the trimming. The candidates for sending the signal are the overlaying

ectoderm and the underlying macromeres. However, on the basis of the lack of specific contact of the posterior end of the germinal band with either tissue, it is probable that they are not responsible for signalling.

The most posterior segment is a specialised segment

The most posterior segment has to be a modified segment because it is carrying the sucker. Although this differentiation happens later than detachment, it is very probable that the most posterior blast cells do not give rise to exactly the same clones as the more anterior ones. In order to evaluate how much is different in this posterior segment, and how early the differentiation can be observe, different methods could be used.

Lineage tracing techniques could allow the descendants of the 32nd and 33rd M blast cells to be specifically labelled and followed. This would provide information about whether any descendants from the 33rd blast cell is required in the 32nd segment (as the labelling experiments done in the mid-body would suggest).

Some genes have been found to be expressed similarly in all segments, namely *en* (Lans et al., 1993) and *twist* (Soto and Weisblat, personal communication). It would be interesting to find out whether the most posterior segment also expressed these genes, and if they do in a similar pattern. Because the most posterior segment is specialised, I would predict that the pattern of expression would be different from that in the mid-body segments.

The models of segment number regulation in the leech.

The cell autonomous model.

In this model, the blast cells are born with an identity, impaired by the teloblast depending on its number of previous cell divisions, or on their environment shortly after birth. This model should be first considered on the basis that the early cell divisions of the leech are highly stereotypic

between individual. A corollary of a stereotypic pattern of cell division is that a specific cell always finds itself in the same environment in the different embryos. A specific cell will therefore always follow the same fate either because it is born with that fate or because the signals sent are always the same.

In the case of the regulation of the number of segments, the high precision of the system (32 segments exactly) and its conservation among a large number of leech species would suggest a very tight genetic control. In effect, it is not possible that a cell has exactly the same relative environment in the Glossiphoniid leeches and the Hirudo leeches (the embryos are direct developers and contain very little yolk).

The arguments against this model come mainly from Shankland's slippage experiment (Shankland, 1984), which showed that the O lineage at least was not cell autonomous in the establishment of its posterior boundary. It is still possible that another lineage would be cell autonomous and would then be signalling the position of the posterior limit to the other lineages. The M lineage is the most likely candidate on the basis of its early detachment, and on the basis that it is the only lineage in direct contact with all the other lineages.

However, cell autonomy for the establishment of the posterior bandlet would require the teloblast to "count" to 32. This is not seen in other systems, and on this basis seems unlikely in the leech.

This model can be tested by doing a bandlet slippage experiment in the M lineage. The cell autonomous model predicts that the cells in the M bandlet slipped posteriorly would not contribute to the germinal band.

The gradient of responsiveness model

This model is a refining of the model presented in Chapter II, and a milder version of the cell autonomous model. The teloblast would not be counting to 32, but to a smaller number. As it divides the teloblast could be

giving away less and less of a molecule. This molecule would then be distributed in an antero-posterior gradient to the blast cells. It is possible to imagine a small number of thresholds could convert the gradient into a limited number of cell states along the body axis. In its most extreme version, the model would predict only 2 states: potential segmental state and potential supernumerary state. In this model again, the precision of the system (32 segments) would suggest that the gradient might not be enough to make a sharp and precise boundary.

The experimental evidence for this model comes from the culture experiment presented in Chapter III. The culture showed that the lack of ectodermal contact cannot be sole responsible for the supernumerary fate adopted by M blast cells. In view of the model, it is possible that the anterior blast cells cultured without ectoderm survived because they had an "anterior" fate that made them relatively insensitive (unresponsive) to their environment. The ectodermal contact would be playing the role of the additional signal required to make a sharp boundary.

This model predicts that posterior slippage of the M blast cell would lead to supernumerary fate, and similarly that posterior blast cells cultured without ectoderm would follow a supernumerary fate distinct from that followed by the anterior ones.

There is a possibility for example, that the cells from the germinal band express cell adhesion molecules (CAMs). These are homophilic molecules, present at the surface of the cells. The cells in the germinal band do not have a uniform surrounding. They adhere to their anterior and posterior neighbour from birth, as seen by the cohesion of the bandlet and the flatten aspect of the cells. As the cells reach the germinal band, they contact blast cells from the other lineages laterally (contact shown by Lucifer Yellow injection). In the model described here, I postulate that the CAMs would be expressed laterally, for the inter-lineage contacts. The CAMs may

be activating through second messengers to turn on any gene responsible for continuing the normal cell cycle. The cells that do not produce these CAMs, or are unable to make contact through them, cannot activate the second messenger pathway, do not divide and die instead. Alternatively, there could be a receptor such as an FGF receptor that would receive a soluble signal and act through a second messenger in the same way as for the CAMs. In this case, the soluble molecule must not be able to diffuse far, as in the slippage experiment, even one cell posterior of the limit dies.

The most anterior blast cells (only those we can be sure were born in culture, and could be traced) might not require such a signal to enable them to divide. They might receive their cue from somewhere else (i.e. at birth, from the teloblast).

The pattern formation candidate genes

Segmentation genes might not be such good candidates after all

The *h* and *ptc* genes were studied as potential candidate for the establishment of the posterior boundary. This was based on the hierarchy found in *Drosophila*: the genes studied are expressed earlier (*h*) or at the same time as the gene *en* (*ptc*), in *Drosophila*. In the leech, *en* is not yet expressed in the cells of the posterior border at the time they detach (Lans et al., 1993), and *h* and *ptc* were therefore likely candidate to be expressed at that time and place.

Even though these molecules cannot be ruled out as candidates for the establishment of the posterior limit, recently published information suggest that the hierarchy of genes found in *Drosophila* is not respected, even in other arthropods (Patel, 1994). It would therefore not be surprising if these genes were not involved in segmentation in the leech.

Homeotic genes could be responsible for establishing the different regions of the body.

The pattern of Homeotic gene expression can be set up as the result of the cells' positional information (in *Drosophila*) or as the result of the cells' genealogy (*C. elegans*). Homeotic genes (Hox genes) have been implicated in the differentiation of body regions in different animals. The Hox genes are expressed in different combinations (or in different cells) along the antero-posterior body axis. The different combinations are thought to give a different identity to the parts of the body. Although these genes, first described in *Drosophila*, have been connected with segmentation (they are downstream of the segmentation genes in *Drosophila*), it is now accepted that they are not directly related to segments, as they are expressed in an antero-posterior fashion in non-segmented animals (e.g. *C.elegans).* Homeotic genes are present in the leech. The best characterised, *lox2*, a *Ubx-labial-* like gene, is expressed in a subset of segments of the leech, at the time blast cells are still being added to the bandlets (Nardelli-Haefliger et al., 1994). It is possible that another of these Hox genes would be present in a subset of segments, with its posterior border coinciding with the segmental/supernumerary boundary.

It is possible that homeotic genes would be regulated by the gradient of molecule established as in the gradient model proposed above. This would reconcile the slippage experiments (Shankland, 1984 and Nardelli-Haefliger et al., 1994) in that the gradient is established at birth (consistent with the fact that *lox2* expression is not regulated), but the establishment of the posterior boundary relies on an additional signal (and is therefore not cell autonomous).

A differential expression library comparing the phenotype of the segmental cells and of the supernumerary cells might, I predict, uncover homeotic genes. These might not be responsible for the establishment for

the boundary but would certainly be good markers of differentiation between segmental and supernumerary fate.

Conclusion

Although the mystery of the regulation of segment number is not solved in the leech, I have uncovered several approaches that can be used to uncover the mechanisms involved. These include finding out which properties of the embryo (morphological and molecular) are kept constant from one embryo to the next at the time of detachment of the bandlets from the germinal band. This would suggest which are the important elements in the regulation of the size of the bandlet. A more systematic approach could involve making a differential expression library to find genes that are specific to the segmental blast cells and genes specific to the supernumerary blast cells.

I do hope that any solution to this problem will prove relevant to other systems, in the same way as certain mechanisms have been found to be used again and again in different organisms and other systems. Perhaps, the regulation of segment number in the vertebrates might occur via similar mechanisms to those of the leech.

Chapter VI. Conclusion

The leech regulates its number of segments through a complex mechanism.

The overall picture that can be drawn from the results presented in this thesis is that the leech regulates its number of segments through a complex mechanism, involving regulation of cell division (of the teloblasts) and of cell death (of specific supernumerary cells).

Regulation of the teloblasts' cell cycle

Primarily, a minimum number of cell divisions of each teloblast is required for attaining the proper number of segments (as seen in Chapter II). The M teloblast undergoes up to 41 rounds of cell division, and so produces up to to 9 supernumerary cells.

The total number of cell divisions of the teloblasts need not be regulated precisely for the correct number to enter the germinal band. It is possible that the teloblast can divide continuously, but that an external factor prevents it from doing so. The action of such a factor could be time-dependent (since the teloblast always takes exactly the same time to produce the segmental blast cells), or position-dependent.

Establishing the position of the limit between the supernumerary and the segmental cells

Secondarily, the supernumerary cells detach from the bandlet, not all at once but in one lineage after the other. This suggests that the limit between supernumerary and segmental cells needs to be set in each lineages independently. This does not mean that the posterior border is independent in the different lineages: the position might be concerted to give rise to such a precise border. However, this rules out the possibilty that one factor

present in the extracellular environment regulates all the teloblasts simultaneously.

Inducing cell death at the limit between segmental and supernumerary cells

Finally, the detachment of the bandlets happens as a shearing between the segmental cells and the supernumerary cells. It is not clear whether this shearing is the result of the establishment of the posterior border or if the shearing of the cells dictates the position of the border, but it is possible that the cell death of the most anterior supernumerary cells is implicated in the detachment of the bandlet. Programmed cell death can be triggered by a variety of mechanisms, and a series of cell death genes (*ced*) have now been found in a different organisms (Driscoli, 1992). It is probable that the leech has *ced* genes homologues, which might be involved in regulation of cell number. If the destruction of the specific cells is the key to establishing the right number of cells in the bandlet, than it is possible that genes involved in triggering cell death (such as *ced3* and *ced 4*) and in protection against cell death (*ced9*) play an important role.

Apart from the most anterior supernumerary cells (those at the border), cell death is not observed to take place in the supernumerary cells before or at detachment, or even soon after. It is even difficult to determine whether these cells really die or are recycled, since they disappear soon after detachment. In any case, their disappearance is more likely to be a cause than a consequence of the trimming of the bandlets.

What the culture says in view of this model.

The culture experiment brought the information that the neighbouring lineages could not be alone in deciding whether a cell should die or not. This suggests that the decision to follow a segmental or a supernumerary pathway of differentiation is the result of both the

environment (neighbouring lineages) and the lineage of each blast cell (born before or after the 32nd round of teloblast division).

The models of segment number regulation.

Positional information models

Positional information has been suggested to play a central role in pattern formation during development (Wolpert, 1989). A positional information model states that a cell will differentiate according to its position within the developmental system. A corollary is that a cell which always assumes the same position in different embryos will always follow the same differentiation.

There are several problems about the suggestion that positional infomation is responsible for the segmental/supernumerary pattern as observed in the leech. First, it requires a gradient, that would span the space between the borders of the field, namely between the teloblasts and the most anterior blast cells. However, there is very wide communication between the cells via gap junction, not only along the antero-posterior axis but also laterally, between the different lineages. If a molecule was freely diffusing antero-posteriorly it would also be diffusing laterally. But the first lineage (namely M) has already set its posterior boundary (and the lineage has therefor reached its final position), when the N lineage is still being born. There is no time-frame during which a gradient infering positional information to all the lineages could be active.

Alternatively to diffusion, a gradient could be set up as the group of teloblasts divide, laying antero-posterior information. The different lineages could set up their own antero-posterior gradient independently. However, the bandlet slippage experiment (Shankland, 1984) argues against this, since posterior border is made to be aligned between the different lineages.

Thirdly, such gradients of positional information are reported to be observed in fields of 1mm or 50 cells maximum, over a period of a few hours (Crick, 1970; Wolpert, 1969). Some of the lineages require 64 primary blast cells in the establishment of the segments, and all the lineages possess at least 50 cells at the time the whole antero-posterior axis is layed down, due to the fact that the most anterior blast cells have already divided many times when the teloblast produces the last segmental blast cells. Moreover, the process of establishing the axis takes a minimum of 70h (time in the M lineage), which is too long to establish a gradient.

Finally, the relative position of the blast cells does vary at the posterior end (single bandlet postition), where the trimming occurs. The cells of similar descent have different position in the different embryos yet they follow their fate according to their descent rather than their position. If they were given positional information, it would be expected that they would follow their fate according to their position.

A cell-interaction model

A model was proposed in Chapter II, where local cell interaction was suggested to play an important role in the decision of the blast cells to become segmental or to die.

There is a possibility for example, that the cells from the germinal band express cell adhesion molecules (CAMs), which are present on the cell surface and which bind in a homophilic manner, which would allow cells from the bandlets to bind to each other laterally. The cells in the germinal band do not have a uniform surrounding. They adhere to their anterior and posterior neighbour from birth, as seen by the cohesion of the bandlet and the flattened appearance of the cells. As the cells reach the germinal band, they contact blast cells from the other lineages laterally (contact shown by Lucifer Yellow injection). It is proposed that the CAMs are expressed laterally, for the inter-lineage contacts. Activation may result in activation of

second messengers that turn on genes responsible for continuing the normal cell cycle (in this model, by default, the blast cells can not keep dividing). The cells that do not produce these CAMs, or are unable to make contact through them, cannot activate the second messenger pathway, do not divide and die instead. Alternatively, there could be a receptor such as a receptor tyrosine kinase that would receive a secreted signal and then trigger a second messenger in the same way as the CAMs. In this case, the molecule must not be able to diffuse far, because as shown in the slippage experiment (Shankland, 1984), even one cell posterior of the limit dies.

The most anterior blast cells (only those we can be sure were born in culture, and could be traced) might not require such a signal to enable them to divide. They might receive their cue from somewhere else (i.e. at birth, from the teloblast).

The number of segments as a constraint

As was suggested in Chapter I, it is possible that the final number of segments in the leech is a consequence of other developmental constraints. Moment (1946) suggested such a constraint in the earthworm : he found that the electrical properties of the worm changed with the number of segments and not with the number of cells. This, he argues, is the reason why the earthworm can only regenerate a specific number of segments.

In the leech, it is possible that such a signal as one caused by an electric current of a maximum intensity, would influence the teloblast and stop it from dividing. In this manner, 32 segments would be the maximum number of segments possible under the constraints. The death and disappearance of the supernumerary blast cells would be a consequence of the germinal band not integrating any more blast cells.

In this model, there is no regulation as such, and the number of segments is the result of a constraint. It could be possible to test the model by measuring the current produced by the leech embryo at different stages

of development, especially between the time the bandlets are at their maximum and after they have been trimmed.

The constraint itself might not be related to the current, and to identify it would require a thorough observation of the factors (morphological and molecular) kept constant from one embryo to another.

The pattern formation candidate genes

Segmentation genes might not be such good candidates after all

The *h* and *ptc* genes were studied as potential candidate for the establishment of the posterior boundary. This was based on the hierarchy found in *Drosophila*: the genes studied are expressed earlier (*h*) or at the same time as the gene *en* (*ptc*), in *Drosophila*. In the leech, *en* is not yet expressed in the cells of the posterior border at the time they detach (Lans et al., 1993), and *h* and *ptc* were therefore likely candidate to be expressed at that time and place.

Even though these molecules cannot be ruled out as candidates for the establisment of the posterior limit, recently published information suggest that the hierarchy of genes found in Drosophila is not respected, even in other arthropods (Patel, 1994). It would therefore not be surprising if these genes were not involved in segmentation in the leech.

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